



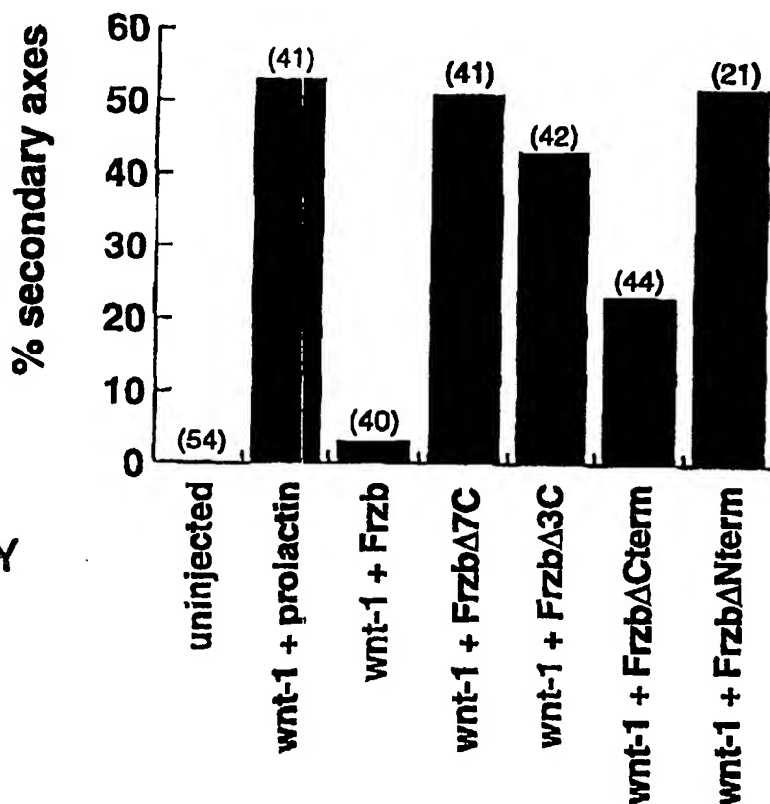
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(54) Title: ISOLATION AND METHOD OF USING TISSUE GROWTH-INDUCING FRZB PROTEIN

(57) Abstract

An isolated cDNA encoding a growth-inducing protein, Frzb, capable of stimulating bone, cartilage, muscle and nerve tissue formation. Frzb binds to and modulates the activity of Wnt growth factors which play a role in various developmental and neoplastic processes. The cDNA and protein sequences of human, bovine and Xenopus Frzb are provided. Production and purification of recombinant Frzb are also described.



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ISOLATION AND METHOD OF USING TISSUE GROWTH-INDUCING FRZB PROTEIN

Field of the Invention

5 The present invention relates to a protein isolated from cartilage capable of inducing skeletal morphogenesis, embryonic pattern formation and tissue specification. More particularly, the invention relates to the Frzb protein which induces *in vivo* cartilage, bone, neural and muscle tissue growth. Frzb also binds to the Wnt family of growth factors and modulates their biological activities

Background of the Invention

10 The discovery and identification of diffusible factors that regulate skeletal morphogenesis have dramatically improved our understanding of the molecular events governing skeletal pattern formation. Genetic studies have confirmed the importance of these differentiation factors in the formation, growth and maintenance of the skeleton (Erlebacher et al., *Cell*, 80:371-378, 1995). Likewise, non-diffusible molecules, including components of the extracellular matrix and cell surface, are essential to patterning processes. One theory proposed for insect systems is that morphogenesis results from the (re)positioning of cells because of inherent characteristics such as differential adhesiveness (Nardi et al., *J. Embryol. Exp. Morphol.*, 36:489-512, 1976). It is presently unknown whether
15 analogous events occur in mammalian skeletal pattern formation.

In *Drosophila melanogaster*, the cuticle contains hairs and bristles arranged in a defined polarity, of which the pattern and orderly alignment reflect the polarity of the wing epidermis (Adler et al., *Genetics*, 126:401-416, 1990). Typically, these structures are aligned in parallel and point in the same direction as the body surface.
20 Several genetic loci associated with epidermal cell polarity have been studied. One of the most thoroughly investigated is the *frizzled* (*fz*) locus. *Frizzled* encodes an integral membrane protein having seven potential transmembrane domains. The *fz* locus is required for cellular response to a tissue polarity signal as well as intercellular transmission of that signal along the proximal-distal wing axis (Vinson et al., *Nature*, 329:549-551, 1987; Vinson et al., *Nature*, 338:263-264, 1989). Mutations of the *fz* locus result in disruption of both cell-autonomous
25 and noncell-autonomous functions of the *fz* gene. Strong *fz* mutations are associated with random orientation of wing hairs, while weaker mutations lead to hair and bristles randomly oriented parallel to neighboring cells with respect to the body axis (Vinson et al., *Nature*, 329:549-551, 1987). *Frizzled* also regulates mirror-symmetric pattern formation in the *Drosophila* eye (Zheng et al., *Development*, 121:3045-3055, 1995).

The rat and human homologs *frizzled-1* and *frizzled-2* (*fz-1*, *fz-2*) have been cloned and are expressed in a
30 wide variety of tissues including kidney, liver, heart, uterus and ovary (Chan et al., *J. Biol. Chem.*, 267:25202-25297, 1992; Zhao et al., *Genomics*, 27:373-373, 1995). Six novel mammalian *frizzled* homologs have now been identified (Wang et al., *J. Biol. Chem.*, 271:4468-4476, 1996), each of which appears to be expressed in a distinct set of tissues during development or postnatally.

The basic form and pattern of the skeleton derived from lateral plate mesoderm are first recognizable when
35 mesenchymal cells aggregate into regions of high cell density called condensations which subsequently differentiate into cartilage and bone, and continue to grow by cell proliferation, cell enlargement and matrix deposition. Published

PCT Application No. WO 96/14335 discloses the isolation, cloning and *in vivo* chondrogenic activity of cartilage-derived morphogenetic proteins (CDMPs) which are members of the TGF- β superfamily. Genetic studies have demonstrated that disruption of condensations results in disturbed skeletal phenotypes (Erlebacher et al., *Cell*, 80:371-378, 1995). In humans, limb development takes place over a four week period from the fifth to the eighth week. The upper limbs develop slightly in advance of the lower limbs, although by the end of the period of limb development the two limbs are nearly synchronized. The most proximal parts of the limbs develop somewhat in advance of the more distal parts.

Recently, the number of secreted factors implicated in both limb and axial patterning has increased steadily (Sive, *Genes Dev.*, 7:1-12, 1993; Dawid, *J. Biol. Chem.*, 269:6259-6262, 1994; Hogan, *Genes Dev.*, 10:1580-1594, 1996). Some of these factors are expressed in the Spemann organizer, the region of the *Xenopus* embryo implicated in specification of the dorsal axis and critical to dorso-ventral patterning of the vertebrate embryo. In contrast, the bone morphogenetic protein BMP-4 and Xwnt-8, a member of the Wnt family of growth factors, are expressed in presumptive ventral mesoderm and endoderm early in gastrulation, and are thought to act as positive ventral inducers (Hogan et al., *supra*; DeRobertis et al., *Nature*, 380:37-40, 1996; Christian et al., *Genes Dev.*, 7:13-28, 1993). Several of these secreted factors are thought to produce their dorsalizing effects by binding to BMP-4 or a related TGF- β class signal and inactivating it. No secreted factor with Wnt binding activity has been identified.

Wnt proteins are implicated in a variety of developmental and neoplastic processes (Nusse et al., *Cell*, 69:1073-1087, 1992; Parr et al., *Curr. Biol.*, 4:523-528, 1994; Moon, *Bioessays*, 15:91-97, 1993). The receptors for these proteins have not been identified. The Wnt family of proteins has been divided into two classes, I and II, based on their ability to induce axis duplication in *Xenopus* oocytes and their transforming activity in mammalian cells. Recently, Frizzled-class proteins were proposed as receptors for the Wnt growth factors (Wang et al., *J. Biol. Chem.*, 271:4468-4476, 1996). This is supported by observations that Wingless protein (Wg), the *Drosophila* prototype of the Wnt family, binds to cells transfected with the *frizzled2* gene (*Dfz2*). Moreover, addition of Wg to cells transfected with *Dfz2* causes increased accumulation of Armadillo, a *Drosophila* homologue of β -catenin, an expected consequence of Wg signaling (Bhanot et al., *Nature*, 382:225-230, 1996). In *Xenopus* embryos, overexpression of rat frizzled-1 (Rfz-1) resulted in recruitment of Xwnt-8 and *Xenopus* dishevelled, a component of the Wnt signaling pathway, to the plasma membrane (Yang-Snyder et al., *Current Biol.*, 6:1302-1306, 1996).

There are few known proteins which induce skeletal morphogenesis, as well as induction of nerve and muscle tissue growth. There are no known secreted proteins which will bind to and modulate the function of the Wnt proteins. Such proteins have tremendous therapeutic applications. The present invention provides such a multifaceted protein.

Summary of the Invention

One embodiment of the present invention is an isolated polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1, 3 or 23.

Another embodiment of the invention is isolated Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7. According to one aspect of this preferred embodiment, at least one acidic, basic, uncharged polar,

nonpolar or aromatic amino acid in the sequence shown in SEQ ID NO: 2, 4 or 7 is replaced with a different acidic, basic, uncharged polar, nonpolar or aromatic amino acid. Preferably, the protein having the amino acid sequence shown in SEQ ID NO: 2 is obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 1. According to another aspect of this preferred embodiment, the protein having the amino acid sequence shown in SEQ ID NO: 4 is obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 3. According to yet another aspect of this preferred embodiment, the protein having the amino acid sequence shown in SEQ ID NO: 7 is obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 23.

Another embodiment of the invention is an isolated polynucleotide encoding a native Frzb protein, the polynucleotide capable of hybridizing to a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1 at 55°C in 3 x SSC, 0.1% SDS.

The present invention also provides an isolated Frzb protein encoded by the polynucleotide described in the preceding paragraph.

Still another embodiment of the invention is an isolated recombinant Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7.

The present invention also provides isolated mammalian Frzb protein having a molecular weight of about 36 kilodaltons.

Another embodiment of the invention is a pharmaceutical composition for inducing cartilage, bone, nerve or muscle growth comprising the isolated Frzb protein encoded by a polynucleotide capable of hybridizing to a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1 at 55°C in 3 x SSC, 0.1% SDS, or a Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7, in a pharmaceutically acceptable carrier.

Still another embodiment of the invention is a pharmaceutical composition comprising an isolated recombinant Frzb protein having the amino acid sequence shown in SEQ ID NO: 2 obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 1, the amino acid sequence shown in SEQ ID NO: 4 obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 3, or encoded by a polynucleotide capable of hybridizing to a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1 at 55°C in 3 x SSC, 0.1% SDS, in a pharmaceutically acceptable carrier. In one aspect of this preferred embodiment, the carrier comprises fibrin glue, freeze-dried cartilage grafts or collagen. The composition may further comprise cartilage progenitor cells, chondroblasts or chondrocytes. Alternatively, Frzb protein may be coated onto or mixed with a resorbable or nonresorbable matrix. In another aspect of this preferred embodiment, Frzb is mixed with a biodegradable polymer.

A further embodiment of the invention is a method of treating a cartilage, bone, nerve or muscle disorder in a mammal in need thereof, comprising the step of administering to the mammal an effective cartilage, bone, nerve or muscle-inducing amount of any of the pharmaceutical compositions described hereinabove at the site of the disorder. Preferably, the administering step is intravenous, intrathecal, intracranial or intramuscular at the site of the disorder. Advantageously, the mammal is a human.

Another embodiment of the invention is a method of stimulating cartilage formation in a mammal, comprising the steps of combining a protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7, or a protein encoded by a polynucleotide capable of hybridizing to a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1 at 55°C in 3 x SSC, 0.1% SDS, with a matrix to produce a product that facilitates administration of the protein; and implanting the product into the body of a mammal to stimulate cartilage formation at the site of implantation. Preferably, the matrix comprises a cellular material. Advantageously, the mixing step additionally comprises mixing of viable chondroblasts or chondrocytes. In another aspect of this preferred embodiment, the implanting is subcutaneous or intramuscular. Preferably, the mammal is a human.

Another embodiment of the present invention are isolated antibodies to the proteins having the amino acid sequences shown in SEQ ID NO: 2 or 4. These antibodies may be either polyclonal or monoclonal.

The present invention also provides a method of modulating Wnt-mediated signaling in a cell, comprising contacting the cell with an effective Wnt-modulating amount of the isolated Frzb protein of Claim 3, a Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7 or an active Wnt-modulating fragment thereof. Preferably, the cell is contacted *in vivo*. Advantageously, the Wnt is Wnt-8, Wnt-1, Wnt-2, Wnt-3, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A or Wnt-7B.

Another embodiment of the invention is a method of modulating Wnt-mediated signaling in a cell, comprising contacting the cell with a recombinant construct comprising the coding region of SEQ ID NO: 1, 3 or 23, or encoding an active Wnt-modulating fragment thereof, operably linked to a heterologous promoter in an expression vector. Preferably, the Wnt is Wnt-8, Wnt-1, Wnt-2, Wnt-3, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A or Wnt-7B.

Still another embodiment of the invention is a method of inhibiting the growth of a Wnt growth factor-expressing tumor in a mammal, comprising the step of contacting the tumor with an effective tumor growth-inhibiting amount of the isolated Frzb proteins described above. In one aspect of this preferred embodiment, the tumor is a mammary or intestinal tumor. Preferably, the mammal is a human.

The present invention also provides a method of inhibiting the growth of a Wnt growth factor-expressing tumor in a mammal, comprising the step of contacting said tumor with a recombinant construct comprising the coding region of SEQ ID NO: 1, 2 or 23 operably linked to a heterologous promoter in an expression vector. Preferably, the construct is injected into the tumor. Alternatively, the construct is systemically administered to the mammal. Advantageously, the expression vector is a plasmid vector, retroviral vector or adenoviral vector.

Yet another embodiment of the invention are isolated antibodies to Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7.

The present invention also provides a method of facilitating tissue growth or repair, comprising the steps of isolating cells from the tissue; introducing a recombinant construct expressing Frzb into the cells; and returning the cells to the tissue. Preferably, the recombinant construct comprises a retroviral vector, adenoviral vector, herpesvirus vector or adeno-associated viral vector. Advantageously, the tissue is cartilage, muscle, bone or neural tissue.

Another embodiment of the invention is a method of identifying a compound which affects Frzb activity, comprising contacting isolated Frzb with the compound; and determining Frzb activity, wherein an increase in activity compared to Frzb alone indicates that said compound is a Frzb activator and a decrease in activity indicates that said compound is a Frzb inhibitor. In one aspect of this preferred embodiment, the determining step comprises an *in vivo* chondrogenesis assay.

Brief Description of the Drawings

Figure 1 shows the nucleotide (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of bovine Frzb. The predicted gene product contains 325 amino acids with a putative signal peptide (boxed). The dashed underline indicates the tryptic peptide sequence used to isolate a cDNA fragment by RT-PCR. Two separate consensus polyadenylation sites are underlined. A "TGA" termination codon is shown in the 5'-untranslated region. The putative signal peptide cleavage site is indicated by the scissors.

Figure 2A shows a comparison between the deduced amino acid sequences of bovine (SEQ ID NO:2) and human (SEQ ID NO: 4) Frzb. The predicted 23 amino acid signal peptide is boxed. The asterisk indicates a potential N-linked glycosylation site. The putative transmembrane region is underlined and bolded.

Figure 2B shows a hydropathy plot of human Frzb from the deduced amino acid sequence. The plot was generated by the GeneWorksTM program using the paradigm of Kyte and Doolittle. Hydrophobic residues are in the upper part of the graph. The arrowhead at the amino terminus indicates the potential signal peptide. The putative transmembrane domain is indicated by a downward arrow. N, C, and P are N-glycosylation, casein kinase 2 phosphorylation, and protein kinase C phosphorylation sites, respectively. The stippled bar underneath the plot represents the *frizzled*-like domain.

Figure 3 shows an amino acid sequence comparison of the N-terminal domain of bovine (amino acids 35-147 of SEQ ID NO: 2) and human (amino acids 35-147 of SEQ ID NO: 4) Frzb, and their homology with amino acids 111-221 of rat fz-1 (SEQ ID NO: 5) and amino acids 53-163 of *Drosophila* frizzled (SEQ ID NO: 6). Identical residues are denoted by shaded boxes. Gaps indicated by hyphens were introduced to optimize sequence alignment. Asterisks indicate conserved cysteine residues. The numbers to the right indicate amino acid residues for each protein.

Figure 4 shows an amino acid sequence comparison between *Xenopus* Frzb (SEQ ID NO: 7), bovine Frzb and human Frzb. Amino acids identical among the three sequences are boxed. A consensus sequence (SEQ ID NO: 8) is shown. The putative signal peptide cleavage site is shown by the pair of scissors.

Figure 5 shows that Frzb can block Wnt-8 signaling across cell boundaries. Ventral *Xenopus* blastomeres were injected with either prolactin (P) or Xfrzb (F) mRNA (50-100 pg per blastomere) as shown at the early 16 cell stage. At the late 16 cell stage, single blastomeres surrounded by those injected previously were injected with Xwnt-8 (W) mRNA (10 pg), and scored for secondary axes. The experiment was performed three times with similar results. Data were pooled for the graph shown in the figure.

Figure 6 is a schematic diagram of the BFrzb and Frzb deletion constructs used for transfection of COS7 cells.

Figure 7 is a graph illustrating the inhibition of Wnt-1-mediated secondary axis formation by Frzb and the effect of various Frzb deletions on its ability to inhibit Wnt-1-mediated signaling. The constructs are shown in Figure 6.

Detailed Description of the Preferred Embodiments

5 The present invention includes polynucleotides encoding Frzb protein isolated from various mammalian tissues, as well as the corresponding protein sequences and variations thereof. Bovine and human Frzb proteins exhibit 94% amino acid identity. An orthologue of Frzb protein, Xfrzb, is also present in *Xenopus laevis* embryos and exhibits about 92% amino acid identity to the corresponding mammalian Frzb proteins in the conserved frizzled-related domain. Bovine articular cartilage extracts were prepared to characterize protein fractions capable of inducing
10 cartilage formation when implanted subcutaneously into rats (*in vivo* chondrogenic activity). Trypsin digestion of highly purified chondrogenic protein fractions followed by polymerase chain reaction (PCR) using degenerate oligonucleotide primers derived from a 30 residue tryptic peptide of the purified protein led to identification of a cDNA encoding a 36 kDa protein. The amino-terminal domain of the deduced amino acid sequence exhibited about 50% amino acid identity to the corresponding region of the *Drosophila* gene *frizzled* which is implicated in the
15 specification of hair polarity during development. Because of its homology to frizzled, the protein was named Frzb.

 The nucleotide and protein sequences of bovine Frzb are set forth in SEQ ID NOS: 1 and 2, respectively. The nucleotide and protein sequences of human Frzb are set forth in SEQ ID NOS: 3 and 4, respectively. The Frzb protein sequences of the invention have the sequences shown in SEQ ID NOS: 2 and 4, or sequence variations thereof which do not substantially compromise the ability of these proteins to induce cartilage, bone, muscle and
20 nerve tissue formation. It will be appreciated that Frzb proteins containing one or more amino acid replacements in various positions of the sequences shown in SEQ ID NOS: 2 and 4 are also within the scope of the invention. Many amino acid substitutions can be made to the native sequence without compromising its functional activity. This assertion is supported by the sequence data shown in Figure 4. Both the mammalian and *Xenopus* proteins have biological activity. The primary sequence divergence, particularly in the carboxyl terminal region of the molecule that
25 contains the exon-intron boundaries, is wider between the amphibian and mammalian forms of Frzb. These sequence differences do not materially alter the biological activity of the protein.

 Variations of these protein sequences contemplated for use in the present invention include minor insertions, deletions and substitutions. For example, conservative amino acid replacements are contemplated. Such replacements are, for example, those that take place within a family of amino acids that are related in the chemical nature of their
30 side chains. The families of amino acids include the basic amino acids (lysine, arginine, histidine); the acidic amino acids (aspartic acid, glutamic acid); the non-polar amino acids (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); the uncharged polar amino acids (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) and the aromatic amino acids (phenylalanine, tryptophan and tyrosine). In particular, it is generally accepted that conservative amino acid replacements consisting of an isolated replacement of a leucine
35 with an isoleucine or valine, or an aspartic acid with a glutamic acid, or a threonine with a serine, or a similar

conservative replacement of an amino acid with a structurally related amino acid, in an area outside of the polypeptide's active site, will not have a major effect on the properties of the polypeptide.

In fact, any protein derivative of SEQ ID NOS: 2 and 4, including conservative substitutions, non-conservative substitutions, mixtures thereof, as well as truncated peptides or sequence variations thereof may be tested as described in the following examples to determine their ability to induce cartilage, bone muscle and nerve tissue. Such routine experimentation will enable the skilled artisan to screen any desired Frzb protein.

A portion of the isolated bovine *frzb* cDNA sequence (SEQ ID NO: 1) was used to screen a human placental cDNA library under high stringency conditions (3 x SSC, 0.1% SDS, 55°C; see Example 3), resulting in isolation of a cDNA (SEQ ID NO: 3) encoding a protein having 94% identity to the bovine protein. The *Xenopus* cDNA sequence corresponding to the conserved frizzled-related region exhibits greater than 80% nucleotide sequence identity to both mammalian *Frzb* genes. Thus, any nucleotide sequence capable of hybridizing to the DNA sequence shown in SEQ ID NO: 1 under these high stringency conditions is within the scope of the invention.

Frzb is recovered in 105,000 x g supernatants of lysates prepared from *Xenopus* embryos or Frzb-transfected mammalian cells, indicating that Frzb is a soluble protein. Both mammalian and *Xenopus* Frzb are secreted from *Xenopus* oocytes injected with the respective mRNAs. In addition, secretion of *Xenopus* Frzb in soluble form was shown by incubation of oocytes with ³⁵S-methionine followed by analysis of culture supernatants by SDS-PAGE. Moreover, mammalian cells transfected with a Frzb expression plasmid secrete Frzb into the culture medium.

Both mammalian and *Xenopus* Frzb were subcloned into the pcDNA3 mammalian expression vector and expressed in *Xenopus* oocytes. This vector contains a CMV promoter which drives expression of the inserted gene. However, other heterologous promoters well known in the art are also contemplated including SV40 and RSV. Bovine and human Frzb were expressed in ATDC5, COS1 and COS 7 cells and partially purified using heparin-Sepharose and Concanavalin A-Sepharose chromatography. The production of Frzb in insect expression systems, particularly baculovirus, is also within the scope of the invention. This protein preparation was used in the functional assays described in the examples presented below. Bovine Frzb was expressed in *E. coli* and purified from inclusion bodies using Ni-NTA affinity chromatography. Many expression vectors suitable for use in eukaryotic expression systems are also within the scope of the present invention, including the LacSwitchTM inducible mammalian expression system (Stratagene, La Jolla, CA) and pcDNA3 (Invitrogen, San Diego, CA).

In situ hybridization and immunostaining of human embryonic sections demonstrate predominant expression surrounding the chondrifying bone primordia and subsequently in the chondrocytes of the epiphyses in a graded distribution that decreases toward the primary ossification center. Transcripts are present in the craniofacial structures but not in the vertebral bodies. Because it is expressed primarily in the cartilaginous cores of developing long bones during human embryonic and fetal development (6 - 13 weeks), has *in vivo* chondrogenic activity and is homologous to *Drosophila frizzled*, Frzb is intimately involved in skeletal morphogenesis via induction of cartilage and bone formation.

As described in the *Xenopus* embryo experiments set forth below (Example 9), both bovine and *Xenopus* Frzb induce formation of secondary body axes which contain neural and muscle tissue, indicating that Frzb is an

important protein component in the molecular pathway leading to initial specification of muscle and nerve in vertebrates. Further, both bovine and *Xenopus* Frzb induces molecular markers for muscle (myo D, actin) and nerve (NCAM) tissue. This was determined by explanting ventral marginal zones during gastrulation (stage 10), followed by grafting onto oocytes expressing Frzb and culturing for an appropriate period of time. Explants were removed and assayed for expression of the particular marker. Untreated ventral marginal zones did not express these markers. These results have been obtained with both injection of mRNA into developing vertebrate embryos and with Frzb protein secreted from *Xenopus* oocytes. Thus, overexpression of the gene encoding Frzb will induce the formation of nerve and muscle tissue in vertebrates.

Wnt proteins are a large class of secreted proteins implicated in a wide variety of differentiation and developmental processes (Cui et al., *Development*, 121:2177-2186, 1995; Bhanot et al., *Nature*, 382:225-230, 1996). When myc-tagged XWnt-8 and Frzb were cotransfected in mammalian cells, Frzb was co-immunoprecipitated with an antibody directed against myc (Example 15). When Frzb mRNA was coinjected with X-Wnt8 mRNA into *Xenopus* oocytes, Wnt-mediated induction of dorsal markers was blocked (Example 13). Thus, Frzb binds and inhibits Wnt-8 during *Xenopus* gastrulation, thus preventing inappropriate ventral signaling in developing dorsal tissues. Because Wnts play critical roles in developmental processes and oncogenesis, Frzb is useful as a modulator of tissue formation and as a tumor suppressor agent.

The cysteine-rich frizzled domain is required for binding of Frzb to Wnt-1 and Wnt-5. While several truncated versions of the frizzled domain co-immunoprecipitate with Wnt proteins, the inhibition of Wnt-1-driven axis duplication in *Xenopus* embryos was abolished upon modification of this domain. The C-terminal domain of Frzb appears to support its inhibitory activity, but is not required. The Frzb-Wnt protein interaction was demonstrated for both Wnt-8 and Wnt-1. Co-injection of Frzb and Wnt-5A did not inhibit the formation of the phenotype characteristic of Wnt-5A-injected *Xenopus* embryos. This suggests that the action of Frzb and other related secreted Wnt binding proteins is not always inhibitory, but may also be stimulatory. Thus, Frzb is capable of modulating Wnt activity. The determination of whether Frzb stimulates or inhibit signaling mediated by a particular Wnt protein can be made using an appropriate assay system for the Wnt protein of interest.

To investigate the specificity of Frzb/Wnt interactions, COS7 cells were co-transfected with Frzb and several HA-tagged Wnt family members (Example 17). In contrast to Frzb which was secreted into the medium in these transfected cells, no Wnt protein was detected in the supernatants. Thus, co-immunoprecipitation experiments were performed with COS7 cell lysates and demonstrated a direct protein-protein interaction between Wnt-1, Wnt-8 and Frzb. As described in Example 18, Frzb co-immunoprecipitated with all of the Wnt proteins tested. Likewise, Wnts co-immunoprecipitated with Frzb. These findings demonstrate that Frzb has sufficient affinity for each of these to allow co-immunoprecipitation. Due to this direct interaction with many members of the Wnt protein family, it is likely that Frzb plays a pivotal role in modulating the activity of all these proteins, as well as Wnt proteins from other mammalian cell types.

Lack of soluble Wnt proteins precluded classical binding studies. Frzb-Wnt interactions were investigated using different washing conditions after immunoprecipitation including a variety of salt concentrations and detergents.

Increasing salt concentrations in the washing did not affect these interactions. Increasing SDS concentrations resulted in the loss of the interaction, although no differences were observed when Wnt-1 (class I) was compared to Wnt-5A (class II). Taken together, no washing conditions could be identified that suggested any differences in the nature of the interactions between Frzb and Wnt-1 or Wnt-5.

5 As discussed in Example 19, the frizzled domain of Frzb is required and sufficient for Wnt binding as shown by immunoprecipitation experiments of COS7 cell lysates co-transfected with Wnt-1 and several deletion constructs and Frzb protein was detected in the supernatant in all instances. The removal of the entire extracellular cysteine-rich N-terminal domain (CRD) resulted in loss of co-immunoprecipitation with the Wnts. Several modifications of the frizzled domain could be made without affecting the outcome of the co-immunoprecipitation experiments.

10 Overexpression of Wnt protein leads to development of mammary tumors in mice (van Leeuwen et al., *Seminars Cancer Biol.*, 6:127-133, 1995; Tsukamoto et al., *Cell*, 55:619-625, 1988). Frzb is particularly useful for systemic or local administration directly into a tumor (e.g. *in situ* tumors), especially for "wnt-driven" tumors such as mammary and intestinal cancers. Determination of whether a tumor is "wnt-driven" can be made by isolating DNA from the tumor and incubating the DNA with a labeled Wnt probe. Frzb can be combined with a pharmaceutically acceptable excipient and injected directly into a tumor. systemically administered, or the nucleotide sequence encoding

15 bovine, human or Xenopus Frzb (SEQ ID NO: 1, 3 and 23, respectively) can be incorporated into an expression vector such as a plasmid, adenoviral vector or retroviral vector by methods well known in the art. These Frzb-containing constructs can be directly injected into a tumor or administered systemically to a mammal.

20 Frzb or constructs encoding Frzb also can be advantageously enclosed in micelles or liposomes. Liposome encapsulation technology is well known. Liposomes can be targeted to a specific tissue, such as tumor tissue, through the use of receptors, ligands or antibodies capable of binding the targeted tissue. The preparation of these formulations is well known in the art (see, for example, Radin et al., *Meth. Enzymol.*, 98:613-618, 1983).

25 Frzb can be used for tissue regeneration, either alone or in conjunction with other morphogenetic proteins, including Wnts, which are implicated in many tissue specification processes. For example, Frzb in conjunction with endogenous Wnt may promote muscle formation and repair. Frzb can also be used to generate tissues or organs *ex vivo* from autologous, immortalized or xenogeneic cell sources.

30 Frzb is contemplated for use in the therapeutic induction and maintenance of cartilage, bone, muscle and nerve tissue. For example, local injection of Frzb as a soluble agent is contemplated for the treatment of subglottic stenosis, tracheomalacia, chondromalacia patellae and osteoarthritic disease. Other contemplated utilities include healing of joint surface lesions (i.e. temporomandibular joint lesions or lesions induced post-traumatically or by osteochondritis) using biological delivery systems such as fibrin glue, freeze-dried cartilage grafts and collagens mixed with Frzb and locally applied to fill the lesion. Such mixtures can also be enriched with viable cartilage progenitor cells, chondroblasts or chondrocytes. Repair or reconstruction of cartilaginous tissues using resorbable or non-resorbable matrices (tetracalcium phosphate, hydroxyapatite) or biodegradable polymers (PLG, polylactic acid/polyglycolic acid) coated or mixed with Frzb is also within the scope of the invention. Such compositions may

35 be used in maxillofacial and orthopedic reconstructive surgery. Frzb can also be used as a growth factor for cells

of the chondrocyte lineage *in vitro*. Cells expanded *ex vivo* can be implanted into an individual at a site where increased chondrogenesis is desired.

The pharmaceutical composition comprising Frzb may also be used to treat or slow neurodegenerative (i.e. Huntington's disease, Alzheimer's disease, spinal cord injuries), myodegenerative (i.e. muscular dystrophy, myasthenia gravis, myotonic myopathies) and osteodegenerative disorders (i.e. osteoporosis, osteitis deformans). A Frzb-containing pharmaceutical composition is administered to an individual in need of facilitated neural, muscle, or bone cell growth in a growth-facilitating amount thereof. The Frzb protein will promote the growth of these tissues. Thus, Frzb is a growth factor or cytokine capable of inducing growth of a variety of tissues. It is also contemplated that Frzb will positively impact the growth of other tissues, including skin and blood vessels. Thus, Frzb-containing compositions may be used for stimulation of wound healing (i.e. lacerations, burns, surgical incisions), promotion of angiogenesis, to prevent rejection in tissue transplantation and as adjuvants to chemotherapy and immunotherapy.

One embodiment of the invention is a pharmaceutical composition comprising the protein shown in SEQ ID NOS: 2 or 4, or sequence variations thereof, in a pharmaceutically acceptable carrier which may be supplied in unit dosage form. Frzb can be administered to an individual in need of facilitated neural, muscle cartilage and bone growth by numerous routes, including intravenous, subcutaneous, intramuscular, intrathecal, intracranial and topical. The compound is combined with a pharmaceutically acceptable carrier prior to administration. Such pharmaceutical carriers are known to one of ordinary skill in the art.

The Frzb compositions for intravenous administration may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. Frzb may be provided as either a bolus or continuous intravenous, intrathecal or intracranial drip infusion. Because the composition will not cross the blood brain barrier, intrathecal (in the cerebrospinal fluid) or intracranial administration is required for treatment of neurodegenerative disorders. The suspension may be formulated according to methods well known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a parenterally acceptable diluent or solvent, such as a solution in 1,3-butanediol. Suitable diluents include, for example, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may be employed conventionally as a solvent or suspending medium. for this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectable preparations.

The Frzb composition may be in soluble or microparticulate form, or may be incorporated into microspheres or microvesicles, including micelles and liposomes.

Contemplated daily dosages of Frzb for parenteral administration to patients with neurodegenerative, myodegenerative, and osteodegenerative disorders are between about 1 μ g and about 100 μ g. Particularly preferred daily dosages are between about 10 μ g and about 50 μ g. This dosage can be administered once per day, or split over 2, 3, 4 or more administrations. Contemplated daily dosages for systemic administration to patients with Wnt-driven tumors or for direct injection into a tumor are between about 100 μ g and 1 mg. The exact dosage can be

determined by routine dose/response protocols known to one of ordinary skill in the art. In a preferred embodiment, administration of Frzb is continued until no further improvement in the particular disorder is observed.

It is also anticipated that the *frzb* polynucleotides of the invention will have utility as diagnostic reagents for detecting genetic abnormalities associated with genes encoding Frzb. Such genetic abnormalities include point mutations, deletions or insertions of nucleotides. Diagnostic testing is performed prenatally using material obtained during amniocentesis or chorionic villus sampling. Any of several genetic screening procedures may be adapted for use with probes enabled by the present invention, including restriction fragment length polymorphism (RFLP) analysis, ligase chain reaction or PCR. Mutations in this gene indicate an increased risk of developmental abnormalities.

Drug screening assays can be used to identify activators or inhibitors of the Frzb protein. For example, Frzb is incubated with a particular drug prior to the *in vivo* chondrogenesis assay described in Example 1 and compared to a control containing Frzb alone. An increase in cartilage growth in the presence of a drug compared to Frzb alone indicates activation of Frzb, while a decrease indicates inhibition of Frzb activity.

The isolation and partial sequencing of a chondrogenic activity present in bovine cartilage is described below.

Example 1

Preparation and activity of articular cartilage extracts

To characterize factors responsible for cartilage inductive activity in articular cartilage, a protein fraction containing potent cartilage inductive activity was isolated as described in PCT Publication No. WO 96/14335. Articular (metatarsophalangeal joints) cartilage extracts were prepared from newborn calves as described (Chang et al., *J. Biol. Chem.*, 269:28227-28234, 1994) to characterize protein fractions with *in vivo* chondrogenic activity. Briefly, tissues were finely minced and homogenized with a Polytron (top speed, 2 x 30 seconds) in 20 volumes 1.2 M guanidine hydrochloride, 0.5% CHAPS, 50 mM Tris-HCl, pH 7.2, containing protease inhibitors and extracted overnight at 4°C as described by Luyten et al. (*J. Biol. Chem.*, 264:13377, 1989). Extracts were concentrated and exchanged with 6 M urea by diafiltration using an UltrasetteTM (Filtron Technology, Inc., MA) and applied to a 0.5 l heparin-Sepharose (Pharmacia/LKB, Piscataway, NJ) column. the column was washed with 5 bed volumes of 6 M urea, 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, then eluted with 2 volumes 1 M NaCl in the same buffer.

In vivo chondrogenic activity was assayed in a subcutaneous implantation model in rats using a collagenous carrier (Luyten et al., *J. Biol. Chem.*, 264:13377-13380, 1989; Luyten et al., *ibid.*). Briefly, a portion of each fraction was assayed by reconstitution with 25 mg guanidine-insoluble collagenous residue of demineralized rat bone matrix according to procedures described by Luyten et al. (*ibid.*). Implants were recovered after 10 days and alkaline phosphatase activity was measured as a biochemical indicator of cartilage and/or bone formation. Implants were also examined histologically for evidence of cartilage formation using standard procedures known to those of ordinary skill in the art.

The 1 M NaCl eluate of articular cartilage, which contained biological activity, was concentrated by diafiltration and applied to a Sephacryl S-200 HR gel filtration column (XK 50/100, Pharmacia/LKB). After molecular sieve chromatography, bioactive fractions were pooled and exchanged into 50 mM HEPES, pH 7.4, containing 0.15 M NaCl, 10 mM MgSO₄, 1 mM CaCl₂ and 0.1% (w/v) CHAPS using MacrosepTM concentrators (Filtron). The

equilibrated sample was mixed with 1 ml ConA Sepharose (Pharmacia-LKB) previously washed with 20 volumes of the same buffer according to the procedure described by Paralkar et al. (*Biochem. Biophys. Res. Commun.*, **131**:37, 1989). After overnight incubation on an orbital shaker at 4°C, the slurry was packed into disposable 0.7 cm ID Bio-Rad columns (Bio-Rad, Hercules, CA) and washed with 20 volumes of the HEPES buffer to remove unbound proteins. Bound proteins were eluted with 20 volumes of the same buffer containing 0.5 M methyl-D-mannopyranoside. The eluate was concentrated to 200 μ l using MacrosepTM concentrators. Macromolecules were precipitated with 9 volumes of absolute ethanol at 4°C overnight. The precipitate was redissolved in 1 ml 6 M urea, 50 mM Tris-HCl, pH 7.4. Bioactive bound protein was mixed with 2 x Laemmli SDS sample buffer (without reducing agents) and analyzed by 12% preparative SDS-PAGE. Gel elution of the separated protein fractions and testing for biological activity was performed as described by Luyten et al. (*ibid.*). Protein fractions from the 36 - 40 kDa region were obtained for bioassay by gel elution following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were found to be chondrogenic.

Primary sequencing data from the bioactive fractions were determined by transfer to PVDF membranes for amino terminal sequencing (Moos et al., *J. biol. Chem.*, **263**:6005-6008, 1988) or to nitrocellulose membranes for trypsin digestion as previously described (Aebersold et al., *Proc. Natl. Acad. Sci. USA*, **84**:6970-6974, 1987; Tempst et al., *Electrophoresis*, **11**:537-553, 1990). Tryptic peptides were separated by reverse-phase high performance liquid chromatography (HPLC) (Epifano et al., *Development*, **121**:1947-1956, 1995), and the sequence of individual peptides was determined using an Applied Biosystems Model 477A sequencer (Applied Biosystems, Foster City, CA) with modifications (Tempst et al., *ibid.*; Tempst et al., *Anal. Biochem.*, **183**:290-300, 1989).

Example 2

Reverse transcriptase-polymerase chain reaction (RT/PCR)

Two degenerate oligonucleotide primers corresponding to the amino- and carboxyl-terminus of the 30 amino acid tryptic peptide 323 (ETVNLVTSAGCLCPPLNVNEEYLIMGYEFP; SEQ ID NO: 9) were used in RT/PCR to clone cDNAs corresponding to peptide 323:

323S: 5'- GA(A/G)AC(A/C/T)GT(C/G)AA(C/T)CT(C/G/T)TA- (C/T)AC(A/C/G/T)-3' (SEQ ID NO: 10); and
323AS: 5'-(A/G)AA(C/T)TC(A/G)TA(A/C/G/T)CCCAT(A/C/G/T)AT-3' (SEQ ID NO: 11)

For RT/PCR, first strand cDNA synthesis was performed with 1 μ g poly(A)⁺ or 5 μ g total RNA prepared from bovine articular chondrocytes using random hexanucleotide primers from the cDNA CycleTM kit (Invitrogen corp., San Diego, CA) or 323AS. 323/323AS primer pairs were used in 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 30 sec. PCR products were purified through a ProbindTM membrane (Millipore), followed by subcloning with the TA CloningTM System (Invitrogen). This yielded a 90 base pair (bp) DNA fragment encoding the proper peptide sequence (dashed underline, fig. 1). The amino acid sequence deduced from the PCR product was the same as the tryptic peptide sequence.

Other tryptic fragments were also sequenced by Edman degradation and had the following sequences:
GVCISPEAIVTA(D or H)GADFPM (SEQ ID NO: 12); QGCEPILIK (SEQ ID NO: 13); QGCEPILICAWPPPLY (SEQ ID NO: 14) and ETVNLVTSAGCLCPPLNVNEEYLIMGYE (SEQ ID NO: 15). SEQ ID NO: 12 containing the D residue corresponds

to amino acids 145-163 of SEQ ID NO: 2. SEQ ID NO: 13 corresponds to amino acids 117-125 of SEQ ID NO: 2. SEQ ID NO: 14 is not found within SEQ ID NO: 2. SEQ ID NO: 15 corresponds to the sequence found within SEQ ID NO: 2 (ETVNLTYSSGCLCPPLNVNEEYLIMGYE; SEQ ID NO: 16) except for position 9 at which there is an alanine in SEQ ID NO: 13 and a serine in SEQ ID NO: 16. The proteins containing these amino acid sequences are most likely structurally and functionally related to the isolated cDNA. These peptides are useful in the design of oligonucleotide probes or in the generation of antisera for nucleic acid hybridization and expression cloning, respectively, of other members of the Frzb protein family. This will allow isolation of other Frzb-related proteins from any vertebrate species.

cDNA clones were isolated and sequenced as described in the following example.

Example 3

Isolation and sequencing of cDNA clones

Bovine articular cartilage total RNA was isolated as described (Luyten et al., *Exp. Cell Res.*, 210:224-229, 1994). Poly(A)⁺ RNA was isolated using the PolyAtractTM magnetic bead system (Promega, Madison, WI). A cDNA library was constructed in a UNIZAPTMXR (Stratagene, La Jolla, CA) starting from bovine articular cartilage poly(A)⁺ RNA. The non-degenerate oligonucleotides designed from the 90 base pair fragment amplified by RT/PCR in Example 2 used to screen the articular cartilage cDNA library were:

323.23: 5'-GCTCTGGCTGCCTGTGTCTCCACTTAACG-3' (SEQ ID NO: 17)

323.40: 5'-CCTCCACTTAACGTTAATGAGGAGTATCTC-3' (SEQ ID NO: 18)

Plaques hybridizing to both oligonucleotides were further purified using standard plaque hybridization procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor laboratory Press, Cold Spring Harbor, NY). A 2.4 kb clone contained a single open reading frame (ORF) with two separate consensus polyadenylation sites and a poly A tail (Fig. 1). A 1.3 kb clone contained a single polyadenylation signal, a short poly A tail and a short 5'-noncoding region. Three other clones lacked the poly A tail but contained longer 5' ends. Because Northern analysis using a bovine cDNA probe revealed corresponding mRNA expression in placenta, a human placental cDNA library was screened to isolate the human orthologue.

Four clones ranging from 1.3 to 1.6 kb were analyzed and all contained the same open reading frame. All clones contained a consensus translation initiation site (Kozak, *J. Biol. Chem.*, 266:19867-19870, 1991) and an in-frame termination codon situated 144 base pairs upstream of the methionine start codon (Fig. 1). The size difference between the bovine and human cDNA inserts (2.4 kb vs. 1.3 kb) is due to a longer 3' untranslated region in the bovine clone (Fig. 1). Based on sequences from these overlapping cDNA clones, the predicted size of both the human and bovine protein is 325 amino acids (Fig. 2A) (36.2 kDa).

The bovine and human amino acid sequences are 94% identical. The deduced protein sequence of both the human and bovine cDNA revealed at least four structural domains (Figs. 1, 2A, 2B). An amino-terminal hydrophobic stretch of 25 amino acids immediately downstream of the initiation methionine likely represents a signal peptide (von Heijne, *Nucl. Acids Res.*, 14:4683-4690, 1986). A second hydrophobic region of 24 amino acids (residues 75-98), which represents a putative transmembrane domain, is followed by a region containing several potential

serine/threonine phosphorylation sites and a serine-rich carboxyl-terminal domain (residues 301-325). Both homologs contain an N-linked glycosylation site at Asn 49, which is amino-terminal of the putative transmembrane domain. A potential C-terminal glycosylation site in the bovine protein was not present in the human homolog.

5 A search of the Gen BankTM data base using the BLAST network service at the national Center for Biotechnology Information (NCBI) (Altschul et al, *J. Mol. Biol.*, 215:403-410, 1990) indicated that Frzb has significant identity (about 50%) in the amino-terminal region (from amino acid 35-147) to *Drosophila frizzled* and rat *fz* proteins (Fig. 3). The homologous region begins shortly after the cleavage site of the predicted signal sequence. The 10 cysteine residues in this region are conserved.

Following isolation of the bovine cDNA, PCR was used to generate a 1 kb fragment containing *XhoI* sites
10 at both ends. This fragment, representing the bovine open reading frame (bORF), was used to screen a human placenta λ gt11 cDNA library (Clontech, Palo Alto, CA). Approximately 7×10^5 plaques from the bovine library and 3×10^5 plaques from the human library were screened. Hybridizations were performed for 24 hours at 42°C in 6 x SSC, 1 x Denhardt's solution, 0.01% yeast tRNA and 0.05% sodium pyrophosphate. The membranes were washed to a final stringency of 3 x SSC, 0.1% SDS at 55°C for 15 minutes (3 x SSC = 50 mM sodium citrate, pH 7.0,
15 0.45 M NaCl).

Sequencing was performed using the dideoxy chain termination method (Sanger et al., *Proc. Natl. Acad. Sci. USA*, 74:5463-5467, 1977) and SequenaseTM Version 2.0 DNA polymerase according to the manufacturer's instructions (United States Biochemical Corp., Cleveland, OH). The sequencing data were obtained by primer walking and from subclones of restriction fragments into pBluescript SKII (Stratagene). Compressions were resolved by
20 performing the sequencing reactions in the presence of 7-deaza-GTP (U.S. Biochemical).

Bovine Frzb was expressed in *E. coli* and purified therefrom as described below.

Example 4

Frzb Protein expression and antibody production

25 The full-length bovine *frzb* gene was subcloned into the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA) under control of the CMV promoter and used to transfect ATDC5, COS-1 (ATCC CRL 1650) and COS-7 (ATCC CRL 1651) cells using the LipofectAMINETM reagent (GIBCO/BRL, Gaithersburg, MD) according to the manufacturer's instructions. A soluble, secreted Frzb protein was obtained from culture supernatants and partially purified by heparin-Sepharose and Concanavalin A-Sepharose chromatography.

30 The bovine *frzb* open reading frame was subcloned in the proper orientation into the *XhoI* site of pET-28a(+) (Novagen, Madison, WI) which contains an amino-terminal stretch of six histidine residues to facilitate purification of the expressed protein as well as a T7 tag for immunodetection. The pET-bORF construct was used in the *E. coli*-based pET SystemTM to obtain bovine Frzb fusion protein. Purification of protein product from inclusion bodies with Ni-NTA affinity chromatography (QIAGEN) was performed using decreasing pH steps according to the manufacturer's
35 instructions. The affinity purified protein was visualized as a major band following Coomassie blue staining after SDS-PAGE. The identity of the fusion product was verified by immunoblotting using a T7 monoclonal antibody.

Rabbits were immunized with Bfrzb fusion protein for 6 months, 250 μ g protein per boost, total of 10 injections. Following immunization, several rabbits were subsequently immunized with a synthetic peptide of 12 amino acids (residues 51-61 of Fig. 1) coupled to keyhole limpet hemocyanin (KLH) through a carboxyl-terminal cysteine. The resulting antisera were screened and titered in immunoblots using the Western-Light PlusTM kit (TROPIX, MA) according to the manufacturer's protocol. Briefly, membranes were incubated overnight in blocking buffer (BF) containing 0.6% I-BLOCKTM (TROPIX) in phosphate buffered saline (PBS) and 0.1% Tween-20. The antiserum was diluted from 1:250 to 1:10,000 in BF. The membranes were washed three times for 5 min in BF after each incubation step. The membranes were incubated with secondary antibody at a dilution of 1:20,000 for 30 min, followed by AVIDIXTM (enzyme conjugate) incubation for 20 min. Blots were developed using the CSPDTM chemiluminescent substrate (TROPIX) and exposed to Kodak XAR-5 film for 1 to 10 min. Antiserum N374-PEP generated against residues 51-61 of Fig. 1 afforded the optimal signal to noise ratio in Western blots and was thus selected for further studies and immunohistochemical staining. This antibody detected a band migrating at the same apparent molecular weight as the Ni-NTA affinity purified protein as determined by Western blot analysis. This method can be used to generate antiserum to human Frzb, as well as any desired immunogenic fragment of bovine or human Frzb.

Monoclonal antibodies to Frzb can also be generated using conventional hybridoma technology known to one of ordinary skill in the art. Briefly, three mice are immunized with 25 μ g recombinant Frzb produced as described in above. Mice are inoculated at 3 week intervals with 20 μ g Frzb per mouse (1/2 subcutaneously and 1/2 intraperitoneally). Serum collected from each animal after the first inoculation reacts with Frzb as determined by immunoprecipitation. Three days after the final inoculation, mice are sacrificed and the spleens harvested and prepared for cell fusion. Splenocytes are fused with Sp2/0 Ag14 myeloma cells (ATCC CRL 1581) with polyethylene glycol (PEG). Following PEG fusion, cell preparations are distributed in 96-well plates at a density of 10^5 cells per well and selected in hypoxanthine/aminopterin/thymidine (HAT) medium containing 10% fetal calf serum and 100 U/ml interleukin-6. The medium is replaced with fresh HAT medium 10 days after plating. To identify hybridomas producing MAbs which recognized Frzb epitopes, hybridoma supernatants are tested for the ability to immunoprecipitate purified Frzb or to detect Frzb by immunoblotting.

As previously discussed, Frzb is a secreted soluble protein; however, to determine whether it also exists in a membrane-associated form, the following cell fractionation study was performed.

Example 5

Cell fractionation

A full length 2.4 kilobase (kb) *Bam*HI-*Xho*I fragment of bovine Frzb (Fig. 1) was cloned into the pcDNA3 expression vector (Invitrogen) to generate the construct pFrzb. COS1 cells (1.6×10^6 initial seeding density) were transfected with 10 μ g of either pFrzb or the control pcDNA3 vector per 100 mm dish using 120 μ l LipofectAMINETM reagent (GIBCO/BRL, Gaithersburg, MD). Transfection was carried out for 6 hours in serum-free OPTI-MEM[®] (GIBCO/BRL). Cells were incubated at 37°C for 72 hours in serum-free OPTI-MEM[®] with daily media changes. Conditioned media were then collected and concentrated 20-fold using a CentriconTM 10 microconcentrator

(Amicon, MA). Cells were scraped from the dishes and resuspended in lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cells were lysed using a syringe and a 25-gauge needle and the resulting lysate was collected. The lysate was centrifuged at 3,000 x g for 10 min to pellet debris, nuclei and non-lysed cells. The resulting supernatant was centrifuged at 100,000 x g for 30 min.

5 The resulting pellet, containing primarily membrane vesicles, microsomes and other particulates, was extracted successively with: 1) 10 mM Tris-HCl, pH 8.0, 6 M urea; 2) 10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 6M urea; 3) 10 mM Tris-HCl, pH 8.0; and 4) 1% SDS in 1% Triton/6 M urea/10 mM Tris-HCl, pH 8.0. After each extraction, samples were centrifuged at 100,000 x g for 30 min. The extracts were then precipitated with an equal volume of 30% trichloroacetic acid (TCA) and re-dissolved in SDS sample buffer. Equal amounts of cytosol, the
10 membrane/particulate fraction and concentrated conditioned media were loaded and separated on 4-20% gradient Tris-glycine gels (Novex, San Diego, CA), blotted to TropifluorTM PVDF membrane (TROPIX) using a GENIETM electrophoretic blotter (Idea Scientific, Minneapolis, MN) and analyzed by immunoblotting as described in Example 4. The primary antiserum (N374-PEP) dilution was 1:1,000. The urea/SDS/Triton extract of the membrane pellet contained most of the Frzb protein. No protein was detected in the supernatants of the transfected cells or in
15 untransfected cells.

 Because the protein sequencing data were obtained from partially purified protein preparations of bovine articular cartilage extracts, similar cell fractionation studies were performed on supernatants and cell extracts of primary bovine articular chondrocyte cultures. Cells were grown to confluence in 100 mm dishes in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), then incubated for 48 hours in serum
20 free OPTI-MEM[®] in the presence or absence of dextran sulfate (250 µg/ml) to improve recovery of soluble protein. Conditioned media and cell layers were processed as described above. Again, most of the protein was detected in the membrane associated fractions. The addition of dextran sulfate did not change this distribution.

 Thus, Frzb exists in both membrane-associated and soluble forms. Recent evidence suggests that the results of cell fractionation studies depend upon the cell or tissue type and are likely related to cell type specific differences
25 in posttranslational proteolytic processing. Frzb is secreted in soluble form in some, but not all, mammalian expression systems. Importantly, Frzb is soluble in frog embryos as described in Example 14. It is possible that Frzb may occur, and act, in both soluble and particulate forms. Nonetheless, the observation that Frzb can be secreted is highly significant in that soluble protein factors are more amenable to production and formulation. In secreted proteins, the signal peptide is cleaved from the preprotein to form the biologically active secreted molecule. In the
30 mammalian cell expression systems used herein, cell lysates contained two Frzb bands as visualized by Western blots, one corresponding to the unprocessed protein containing the signal peptide, and one corresponding to the processed protein lacking the signal peptide. When Western blots were performed on a clarified lysate of *Xenopus* embryos, a single protein band was observed.

 Localization of mRNA encoding Frzb in human embryos was determined by *in situ* hybridization as described
35 below.

Example 6

In situ hybridization

Serial sections of human embryos representing various stages of development were used for *in vitro* hybridization to explore the pattern of Frzb expression during embryonic development. Tissues from human embryos ranging from 6 to 13 weeks of gestation, estimated on the basis of crown-rump length and pregnancy records, were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), embedded in paraffin, cut serially at 5-7 μ m and mounted on salinated slides. These tissues were obtained from legally sanctioned procedures performed at the University of Zagreb Medical School, Zagreb, Croatia. The procedure for obtaining autopsy materials was approved by the Internal Review Board of the Ethical Committee at the University of Zagreb School of Medicine and the Office of Human Subjects Research of the National Institutes of Health, Bethesda, MD. *In situ* hybridization was performed as described previously (Pelton et al., *Development*, 106:759-767, 1989; Vukicevic et al., *J. Histochem. Cytochem.*, 42:869-875, 1994). Briefly, after a short prehybridization, sections were incubated overnight at 50°C in 50% formamide, 10% dextran sulfate, 4 x SSC, 10 mM dithiothreitol (DTT), 1 x Denhardt's solution, 500 μ g/ml freshly denatured salmon sperm DNA and yeast tRNA with 0.2 - 0.4 ng/ml ³⁵S-labeled riboprobe (1 x 10⁹ cpm/ μ g) in a humidified chamber. Since the bovine Frzb open reading frame contained *Xho*I sites at both ends, this fragment was subcloned in both sense and antisense directions into the *Xho*I site of pBluescript SKII- vector and riboprobes were made using T7 RNA polymerase according to the manufacturer's instructions (Novagen). After hybridization, the sections were washed to a final stringency of 0.1 x SSC, 65°C for 2 x 15 min. After dehydration in a graded ethanol series containing 0.3 M ammonium acetate, slides were covered with NTB-2 emulsion (Kodak) and exposed for 1 - 3 weeks. The slides were then stained with 0.1% toluidine blue, dehydrated, cleared with xylene and mounted with Permount.

Between 6 and 13 weeks, no hybridization was detected in most organs, including kidney, heart, muscle, intestine, liver, brain and lung. In contrast, strong hybridization was seen in the developing appendicular skeleton. At six weeks, Frzb transcripts were clearly visible surrounding the early cartilaginous rudiments of the developing limbs, as shown in the distal parts of the upper limb. Hybridization was apparent between neighboring areas of cartilaginous condensation in developing long bones. Subsequently, expression appeared within the cartilaginous cores of developing long bones. This was apparent in the proximal parts of the upper limb, which are more advanced in developmental state than the distal parts. Frzb was also present in the putative limb primordia, thereby bridging the expression data obtained in early development to the localization in developing limbs. Additional experiments in developing limbs have revealed expression in the precartilaginous condensations and subsequently in the future joint interzones.

In addition, Frzb was detected in the cartilage anlagen of several craniofacial bones and the epiphysal ends of the rib cage, while no signal was detected in the vertebral bodies at 6 weeks. At 13 weeks of gestation, Frzb transcripts were present in early chondroblasts of the tarsal bones of the foot, the carpal bones of the hand and the epiphysis of long bones. A striking feature of the expression pattern at this developmental stage was the

presence of a graded distribution, most prominent in the phalanges. The highest level of expression was observed at the epiphyses of long bones and at the periphery of cuboidal bones. The expression level then decreased with the appearance of chondrocyte hypertrophy and vascular invasion and appeared to be absent in the primary centers of ossification. Interestingly, at this stage of development, several layers of chondroblasts adjacent to the joint space did not show detectable transcripts. In sharp contrast to the prominent expression observed in other skeletal structures, no expression was apparent in the vertebral bodies at the stages examined.

A *Xenopus laevis* orthologue of Frzb (Xfrzb) was isolated as described below.

Example 7

Isolation of Xfrzb cDNA

The primers 5'-TGGAACATGACTAAGATGCCC-3' (SEQ ID NO: 19) and 5'-CATATACTGGCAGCTCCTCG-3' (SEQ ID NO: 20) were used to label a region of the bovine Frzb cDNA sequence having a high degree of sequence identity to related genes from human and avian sources. 106 plaques from a Stage 20 *Xenopus* cDNA library prepared in ISH-lox (Novagen, Madison, WI) were screened at low stringency (final stringency 35-BOC in 20 mM Na₂HPO₄, pH. 7.2, 1 mM EDTA, 1% SDS) and purified plaques were characterized by direct sequencing (Wang et al., *BioTechniques*, 130-135, 1995). One 498 bp clone was 92% identical to a region of the bovine sequence. Two oligonucleotides, 5'-GTCTTTTGGGAAGCCTTCATGG-3' (SEQ ID NO: 21) and 5'-GCATCGTGGCATTTCAC TTTC A-3' (SEQ ID NO: 22), corresponding to the 5' and 3' regions of this partial length clone, were used to screen duplicate lifts from a stage 13 library (Richter et al., *Proc. Natl. Acad. Sci. USA*, 8086-8090, 1988). Plaques that hybridized to both oligonucleotides were further analyzed. Several clones containing a complete open reading frame were identified and sequenced. Two closely similar clones were isolated and one of these was chosen for further study. The nucleotide and deduced amino acid sequences of this Xfrzb clone is shown in SEQ ID NOS: 23 and 7, respectively.

Xfrzb shares several features common to the mammalian proteins, including a consensus site for asparagine-linked glycosylation, a conserved cysteine-rich domain characteristic of Frizzled proteins, and a carboxyl terminal motif (amino acids 244-293) that appears homologous to the netrin-specific carboxyl-terminal domain of *C. elegans* unc-6 (Wadsworth et al., *Bioessays*, 16:355-362, 1996).

Expression of Xfrzb was analyzed by *in situ* hybridization as described in Example 6. Expression begins early in gastrulation and continues as the embryo matures. Thus, it is present when many of the most important events in the establishment of the overall body plan of the developing embryo occur. It is expressed initially in the organizer region, extending beyond it during gastrulation. At the end of gastrulation, expression in this region abruptly ceases and then appears in primordial head mesoderm. Expression then becomes more localized, ultimately to a region corresponding to the developing pituitary gland. These observations are consistent with an important role in the induction of the nervous system and axial musculature, from which the majority of skeletal muscle is derived. Its expression in the pituitary suggests a prominent role in defining anterior mesodermal structures, including the pituitary itself.

Example 8**Immunohistochemical staining**

5 Tissue sections were stained using the Vectastain[®] elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. All embryos were embedded in JB-4 resin (Polysciences, Warrenton, PA). For conventional histological analysis, 1-3- μ m sections were cut and stained with hematoxylin and eosin. Before staining, tissue sections were pretreated with chondroitinase ABC for 1 hour. The sections were blocked with PBS and 10% goat serum for 30 min, then incubated for 1 hour with primary antiserum (N374-PEP) at a dilution of 15 μ g/ml in PBS containing 0.5% goat serum. In the controls, the primary antibody was replaced with normal pre-immune rabbit serum or secondary antibody alone.

10

Immunohistochemical staining confirmed the presence of protein in developing skeletal structures, appearing within the cartilaginous cores of the developing long bones. The graded mRNA expression pattern detected by *in situ* hybridization, most prominent in the phalanges, was paralleled by the protein distribution.

Example 9

15 **Ectopic expression of Frzb in *Xenopus* embryos**

Ectopic expression in developing *Xenopus* embryos induced formation of secondary body axes which contained neural and muscle tissue, but no notochord. This assay is an extremely stringent and specific test for the ability of a gene product to initiate a complex program of developmental events and indicates that Frzb can initiate the synthesis of nerve and muscle tissue. Further, overexpression of Xfrzb in explants fated to develop into ventral

20 tissue induced molecular markers of muscle and nerve tissue.

Ultraviolet irradiation interrupts the normal mechanism for establishment of the dorso-anterior body axis, so that treated embryos did not develop dorsal structures (i.e. head, somites, neural tube, notochord) or the tissues comprising them. When irradiated enzymatically defolliculated embryos were injected with 50 μ g mRNA encoding Xfrzb, a body axis was restored. The reconstituted axis contained a neural tube and dysmorphic somites, but no notochord. This experiment is an even more demanding test of the ability of a protein to initiate a complex developmental program. If a truncated construct, containing only the putative extracellular and transmembrane regions of the molecule, was used for injection with mRNA at the two cell stage of one blastomere, one half of the embryo appeared to develop normally, while the other was devoid of both muscle and neural tube; the notochord was normal bilaterally. This study evaluated the effects of ablating the function of Xfrzb, based on the premise that the defective molecule could act as a competitive inhibitor of endogenous Frzb. The effect produced by the defective Frzb was in essence the converse of what is observed if the unmodified gene is overexpressed.

As an initial test of the ability of Frzb to play a role in patterning of the vertebrate embryo, the effects of overexpression and ectopic expression of Frzb in developing *Xenopus* embryos were evaluated.

All embryos were embedded in JB-4 resin (Polysciences, Warrenton, PA). for conventional histological analysis, 1-3 μ m sections were cut and stained with hematoxylin and eosin; 10-20 μ m sections were taken from embryos stained by in situ hybridization. Darkfield images of embryos were photographed with low angle oblique illumination and a Zeiss Stemi-6 dissecting microscope. Embryos cleared with benzyl alcohol/benzyl benzoate and the histological sections in Example 10 was photographed under diascope illumination with a Nikon FXA microscope. the sections in Example 11 were photographed under multiple oblique illumination (Edge Scientific, Santa Monica, CA).

Example 10

Dorsalization of embryos by Frzb

Enzymatically defolliculated single ventral blastomeres at the 4 cell stage were injected with 50 ng bovine Frzb (Bfrzb) mRNA and cultured with oocyte Ringer's solution as previously described (Kay, *Methods Cell Biol.*, 36:657-669, 1991). Frogs and their embryos were maintained and manipulated using standard methods (Gurdon, *Methods Cell Biol.*, 16:125-139, 1977). mRNA injection was performed as described previously (Moos et al., *Development*, 121:4293-4301, 1995). Dorsal and ventral blastomeres were identified by size and pigment variations. Lithium treatment was for 1 hour at 0.1 M (Kao et al., *Dev. Biol.*, 127:64-77, 1988). UV irradiation was performed with a StratalinkerTM (Stratagene). Animal cap explants were cultured in 0.7-1x Marc's Modified Ringer's solution (Kay, *supra.*). Activin was a gift from the National Cancer Institute and bFGF was from GIBCO/Life Technologies (Gaithersburg, MD).

Injection of Bfrzb mRNA into single ventral blastomeres produced duplicated posterior dorsal axes reproducibly. Muscle and neural tissues were apparent in frontal sections taken from these embryos, but notochord was absent. The frequency of axis duplication was approximately 15% (24/159; four independent experiments) with Bfrzb and somewhat less with the *Xenopus* gene. The difference may be due to the presence of a consensus translation initiation site (Kozak, *J. Biol. Chem.*, 266:19867-19870, 1991) in the bovine, but not the amphibian sequence. The phenotypes were identical in either case. When Frzb was injected into UV-irradiated embryos which

are incapable of axis formation, dorsal axes were partially rescued in approximately 56% (37/66; three independent experiments). The rescued axes contained muscle and neural tube, but no notochord. Nevertheless, overexpression of Frzb in animal cap explants did not induce markers for mesoderm (Brachyury (Xbra)), neural tube (NCAM), or somites (muscle actin) (not shown).

5

Example 11

Expression of Xfrzb in developing Xenopus embryo

The Xfrzb open reading frame was subcloned into pCR-Script (Stratagene) to generate probes for in situ hybridization. Both Bfrzb and Xfrzb were subcloned into pSP64R1 (Dr. S. Sokol, Harvard University) for mRNA injection experiments. The pSP64T-Xwnt-8^{myc} plasmid used for mRNA injections and *in vitro* translation and the
 10 CSKA-X8 expression plasmid are described by Christian et al. (*Genes Dev.*, 7:13-28, 1993). A pGEM-5R-Xwnt-8 plasmid (Smith et al., *Cell*, 67:753-765, 1991) was used to generate probes for in situ hybridization. *In vitro* transcription was performed using mMessage mMachine or MEGAscript kits from Ambion (Austin, TX). The plasmid pLNCWnt1HA, containing the open reading frame of mouse Wnt1 and a hemagglutinin (HA) tag near the C-terminus, was provided by Dr. J. Kitajewski (Columbia University). The Xlrf25 plasmid used for in situ hybridization analysis
 15 of MyoD is described by Scales et al. (*Mol. Cell. Biol.*, 15:1515-1524, 1995). The pfrzb expression plasmid is described by Hoang et al. (*J. Biol. Chem.*, 271:26131-26137, 1996). In situ hybridization was performed as outlined by Harland (*Methods Cell Biol.*, 36:685-695, 1991), with modifications as described by Moos et al. (*Development*, 121:4293-4301, 1995).

Xfrzb expression first became apparent in the late blastula (stage 9) by in situ hybridization. In early
 20 gastrulas (stage 10), mRNA expression was most apparent in the Spemann organizer. In later gastrulas (stage 10.5-11), there was expression in the blastopore lip that extended beyond the organizer as the blastopore lip progressed ventrally. At about stage 11, Xfrzb expression appeared in the dorsal midline. Examination of cleared embryos and corresponding histological sections revealed that this expression was in the involuted mesoderm which is thought to convey signals to the overlying neuroectoderm that participates in specification of the nervous system. Near the
 25 onset of neurulation, posterior expression was markedly reduced, and expression in the prechordal plate became apparent. The field of expression was then restricted progressively, stabilizing in the putative pituitary and posteriorly in the vicinity of the proctodeum. Thus, Xfrzb is expressed at the appropriate time and place to participate in specification of the body axis. These results are consistent with RT-PCR analysis.

Example 12

30

Immunoblotting, immunoprecipitation and *in vitro* translation

Embryos and oocytes were lysed by sonication on ice in 40 mM Tris base, 10 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride (PMSF) in a volume of 10 μ l/embryo or oocyte. In some experiments, 20,000 x g supernatants were extracted with an equal volume of 1,1,2-trichlorofluoroethane (Evans et al., *Methods Cell Biol.*, 36:117-132, 1991). *In vitro* translations were performed in the presence of ³⁵S-methionine with nuclease-treated
 35 rabbit reticulocyte lysate and canine pancreatic microsomal membranes (Promega, Madison, WI) according to the manufacturer's instructions. β -lactamase mRNA supplied with the kit was used as a positive control for translation

and processing and as a negative control for nonspecific protein-protein interaction. SDS-PAGE was performed using Novex 10% Nu-PAGE gels (Novex, San Diego, CA). Samples from embryos were precipitated with methanol/chloroform (Wessel et al., *Anal. Biochem.*, 138:141-143, 1984) prior to analysis. For metabolic labeling studies, gels were dried onto a single sheet of cellophane and imaged with BioMax MR2 film (Kodak) or a phosphor screen (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation was performed according to standard procedures (Harlow et al., *Antibodies: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Antiserum N374-PEP against Xfrzb was prepared as described (Hoang et al., *supra.*). The clone 9E10 monoclonal antibody (Boehringer, Indianapolis, IN) was used for precipitation or detection of the c-myc epitope and hybridoma supernatant containing the 12CA5 monoclonal antibody was used for precipitation of the HA epitope. Immunoblot analyses of separated proteins were performed following transfer to nitrocellulose membranes using 1:20,000 dilutions of primary antisera and 1:100,000 dilutions of peroxidase-conjugated secondary antibody. Bands were detected with the Super Signal Ultra peroxidase substrate (Pierce, Rockford, IL).

Endogenous Xfrzb could be detected in early gastrulas (stage 10) and all subsequent stages analyzed by immunoblot analysis. Xfrzb expression was unaffected by bFGF, enhanced by activin or lithium, and suppressed by UV irradiation as described for other genes expressed in the Spemann organizer (Slack, *Curr. Biol.*, 4:116-126, 1994; Kao et al., *Dev. Biol.*, 127:64-77, 1988).

Example 13

Blocking of Wnt-8 signaling *in vivo* by Frzb

When Xwnt-8 mRNA is injected during early embryogenesis, secondary dorsal axes with complete head structures are induced reliably (Smith et al., *supra.*; Sokol et al., *Cell*, 67:741-752, 1991). This phenomenon was used as an *in vivo* assay for Xwnt-8 activity. When prolactin mRNA was coinjected with Xwnt-8 message, 71% of the embryos (27/38) developed secondary axes. In contrast, when the prolactin mRNA was replaced by an identical amount of Frzb mRNA, axis duplications were suppressed (0/32 for Xfrzb; 1/36 for Bfrzb). Uninjected embryos did not display axial abnormalities.

A Xwnt-8 expression plasmid under the control of the cytoskeletal actin (CSKA) promoter induces the ventrolateral marker Xpo (Sato et al., *Development*, 112:747-753, 1991) and suppresses induction of the dorsal marker goosecoid in activin-treated animal cap explants (Hoppler et al., *Genes Dev.*, 10:2805-2817, 1996). This effect was blocked completely in caps overexpressing a dominant-negative Xwnt-8. Our results confirmed that Xpo expression could be increased by Xwnt plasmid in activin-treated animal cap explants. Importantly, this effect was blocked by XFrzb.

ADMP is a Spemann organizer specific marker that is induced by activin in animal cap explants (Moos et al., *supra.*). Induction of ADMP by activin was suppressed in explants injected with Xwnt-8 plasmid; this suppression was rescued by Xfrzb. Frzb overexpression did not affect the expression level of Xwnt-8. Thus, Frzb appears to exert its dorsalizing effects by inhibiting the action of Xwnt-8. In a related experiment, the CSKA-Xwnt-8 plasmid was injected into dorsal blastomeres with or without Xfrzb mRNA. In this assay, CSKA-XWnt-8 plasmid produced

head defects (64/80 embryos, three independent experiments), as described previously (Christian et al., *Genes Dev.*, 7:13-28, 1993). However, if Xfrzb mRNA was coinjected with the CSKA-Xwnt-8 plasmid, these defects were not observed (0/81 embryos).

5 induction of Siamois and Xnr3 proteins (Lemaire et al., *Cell*, 81:85-94, 1995; Smith et al., *Cell*, 82:37-46, 1995) in animal cap explants injected with Xwnt-8 mRNA has been used to assay Xwnt-8 signaling (Carnac et al., *Development*, 122:3055-3065, 1996; Yang-Snyder et al., *supra.*). Both Xfrzb and Bfrzb blocked the induction of these genes by Xwnt-8.

Example 14

Cell fractionation of Xenopus embryos and action across cell boundaries

10 The subcellular distribution of Xfrzb expressed in vivo was analyzed. Endogenous Xfrzb protein was found in 105,000 x g supernatants isolated from Xenopus embryos, but could not be detected in cell pellets, in contrast to Bfrzb (Example 5). Further, Frzb was secreted by oocytes injected with Frzb mRNA. The apparent molecular weight of 33 kDa is consistent with removal of the putative signal sequence. Proteolytic processing likely accounts for the difference in molecular weight between secreted Frzb and Frzb contained in oocyte lysates.

15 To determine whether Frzb could act across cell boundaries, an experimental design used to study the dominant-negative Xwnt-8 (Hoppler et al., *supra.*) was used. Xfrzb reduced the percentage of secondary axes induced by Xwnt-8 from 52% (46/88) to 10% (5/49) when the two mRNAs were injected into different cells. This indicates that the effects of Frzb on Xwnt-8 occur following secretion.

Example 15

Direct interaction of Frzb and X-Wnt8 proteins

20 Direct interaction between Frzb and X-Wnt8 proteins was demonstrated in two systems: rabbit reticulocyte lysate containing canine microsomal membranes, and transfected COS cells. COS 7 cells (1.6×10^6 initial seeding density) were transfected with 5 μ g of pfrzb (see Example 5) or pLNCWnt1HA (see Example 11), or co-transfected with 4 μ g pfrzb and pLNCWnt1HA in 100 mm dishes using 30 μ l LipofectAMINETM (Life Technologies, Inc., Gaithersburg, MD). Transfections were performed for 6 hours in serum-free Opti-MEM 1[®] (Life Technologies). Thereafter, cells were incubated for 18 hours in media containing 10% fetal bovine serum. Subsequently, cells were cultured at 37°C for 24 hours in serum-free Opti-MEM 1[®]. Cells were extracted for 30 minutes on ice with 50 mM Tris-HCl, 150 mM NaCl, 1.0% NP-40, 0.5% Deoxycholic acid and 0.1% SDS and centrifuged at 12,000 x g for 5 minutes. Supernatants were saved for immunoprecipitation.

30 Xwnt-8^{myc}, Bfrzb, Xfrzb, and the β -lactamase control mRNA were all translated and processed *in vitro*, either alone or in the following combinations: Wnt-8 + Frzb, Wnt-8 + β -lac; Frzb + β -lac. As expected, the anti-myc antibody precipitated Xwnt-8^{myc} but not β -lactamase, Xfrzb or Bfrzb. Conversely, the 374-PEP antiserum, which recognized both mammalian and amphibian Frzb in immunoblots, precipitated both Xfrzb and Bfrzb, but neither Xwnt-8^{myc} nor β -lactamase. However, when Xwnt-8^{myc} and Frzb were cotranslated, both proteins were precipitated
35 by either the myc-specific 9E10 monoclonal antibody or the 374-PEP antiserum. Identical results were obtained with Bfrzb. Neither reagent precipitated β -lactamase cotranslated with Frzb or Xwnt-8^{myc}.

These results were further supported by experiments in which COS7 cells were co-transfected with expression plasmids encoding Bfrzb and an HA-tagged murine Wnt-1, which belongs to the same functional class as Xwnt-8 (Nusse et al., *Cell*, 69:1073-1087, 1992). Cell lysates were immunoprecipitated with an anti-HA antibody, immunoblotted and probed with the Frzb-specific 374-PEP serum. Frzb protein was detected only in lysates from cells transfected with both Frzb and Wnt-1.

Example 16

Frzb blocks MyoD expression

Xwnt-8 was implicated in somite development using a carboxy-terminal deletion construct which acted in a dominant negative fashion (Hoppler et al., *supra.*). Because our data suggested that Frzb could also act as a Wnt inhibitor, we evaluated its effects on somite formation and MyoD expression, both of which are suppressed by the dominant negative Xwnt-8. When Xfrzb mRNA was injected radially into all blastomeres at the four cell stage, trunk development was grossly abnormal, resembling that seen in embryos overexpressing the dominant negative Xwnt-8. Furthermore, Xfrzb blocked MyoD expression both in gastrulating embryos and in activin-treated animal cap explants.

At first glance, the ability of Frzb to induce partial dorsal axes or suppress MyoD expression appear to be incompatible. However, these observations can be reconciled by consideration of the cellular context in which overexpression of Frzb occurs. Ectopic gene expression may generate a secondary axis by a direct inductive effect, or indirectly by inhibition of a ventralizing signal. Frzb blocks the actions of Xwnt-8 as described in Example 13, but does not induce mesoderm, muscle or neural tissue when overexpressed in animal cap explants which do not express Xwnt-8 (Example 10). The dorsalizing actions of Frzb are thus likely to be indirect, resulting from inhibition of the ventralizing effects of Xwnt-8.

Local overexpression of a molecule acting in such an indirect manner produces different effects than generalized overexpression. Injection of Frzb into a single blastomere within the expression domain of Xwnt-8 is expected to block its ventralizing activity locally. Generation of a partial dorsal axis by Frzb (Example 10) is consistent with this prediction. On the other hand, generalized overexpression will block all actions of Xwnt-8 throughout the embryo, including both its ventralizing activity and its effects on somite formation.

Recently, a dominant-negative Xwnt-8 was shown to suppress development of the trunk and somites (Hoppler et al., *Genes Dev.*, 10:2805-2817, 1996). When Frzb was overexpressed using an identical protocol (all blastomeres at the four cell stage), the same phenomenon was produced (Example 16). Thus, the induction of muscle tissue by local overexpression of Frzb in one type of experiment and suppression of somite development by generalized overexpression in another are compatible findings consistent with the conclusion that Frzb acts through inhibition of Xwnt-8 signaling.

To investigate the specificity of Frzb/Wnt interactions, COS7 cells were co-transfected with Frzb and several HA-tagged Wnt family members as described below.

Example 17**Transient transfection of COS7 cells**

Plasmids used for transfection of COS7 cells were as follows. Δ c-term was made by deletion of most of the C-terminal region of Frzb from amino acids 160 to 316. Δ CRD was made by deletion of most of the frizzled domain of Frzb from amino acids 39 to 145 including nine conserved cysteines (C43-147). Δ 7C was made by deletion in the frizzled domain of amino acid 79 to 149 including seven conserved cysteines (C88-147). Δ 2C was made by a deletion in the frizzled domain from amino acid 124 to 149, including two conserved cysteines (C136-147). Δ 57-95 was made by deletion in the frizzled domain from amino acids 57 to 95 which interrupted the hydrophobic structure in the frizzled domain. These constructs are shown in Fig. 6. pFrzb-FLAG was made by replacement of the last seven residues of Frzb by a FLAG-tag. All constructs were subcloned into pCDNA3 (Invitrogen). The following plasmids carried ten mouse Wnt gene family members: pLNCW1-HA, pLNCW2-HA, pLNCW3A-HA, pLNCW3B-HA, pLNCW4-HA, pLNCW5A-HA, pLNCW5B-HA, pLNCW6-HA, pLNCW7A-HA and pLNCW7B-HA.

COS7 cells (1.6×10^6 initial seeding density) were transfected either with 5 μ g plasmid DNA, or co-transfected with 4 μ g for each plasmid per 100 mm dish using 30 μ l LipofectAMINETM reagent (GIBCO/BRL). Transfections were carried out for 6 hours in serum-free Opti-MEM I[®] (GIBCO/BRL). Equal amounts of 10% FBS in Opti-MEM I[®] were added to the transfections and the cultures were continued for 18 hours. The cells were then incubated at 37°C for 24 hours in serum-free Opti-MEM I[®]. The cells were extracted for 30 minutes on ice with 50 mM Tris-HCl, 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid and 0.1% SDS.

Example 18**Immunoprecipitations with HA and FLAG antiserum**

Fifty μ l of protein A-agarose (Behringer Mannheim GmbH, Germany) was incubated with 100 μ l hybridoma supernatant of anti-HA antibody 12CA5 in 450 μ l of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl by rotating overnight. 100-400 μ l of the cell lysates from transfected COS7 cells and 0-300 μ l of RIPA buffer were added to the mixture to a final volume of 1 ml and incubation was continued for another hour. The agarose beads were washed by centrifugation at 12,000 x g for 20 seconds, followed by rotation for 20 minutes twice in 1 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, pH 7.5; twice in 1 ml 50 mM Tris-HCl, pH 7.4, 500 mM NaCl and once in 50 mM Tris-HCl, pH 7.4, respectively, and centrifuged at 12,000 x g. After the last wash and centrifugation, pellets were suspended in 30-50 μ l of 2 x Laemmli sample buffer with 4% β -mercaptoethanol, boiled, separated on 4-20% gradient Tris/Glycine gels (Novex), blotted onto ImmobilonTM-P membranes (Millipore) and analyzed by immunoblotting.

Membranes were blocked for 30 min in blocking buffer (BF) consisting of 1 M Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween-20 and 4% BSA. The primary antiserum (N374-PEP) was incubated with the membranes in 1/10 BF and 9/10 TBST buffer (10 mM Tris-HCl, pH 7.5, 0.1% Tween, 150 mM NaCl) at a dilution of 1:2,500. The membranes were washed four times for 5 min in TBST after each incubation step. The membranes were then incubated with the secondary antibody at a dilution of 1:10,000 for 60 minutes. Blots were developed using the

Frzb co-immunoprecipitated with all the Wnts tested (Wnt-1, Wnt-2, Wnt-3A, Wnt-3B, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A and Wnt-7B). Likewise, using a flag-tagged *BFrzb* cDNA, Wnts co-immunoprecipitated with Frzb. Thus, Frzb has sufficient affinity for each of these Wnt proteins to allow co-immunoprecipitation.

10

Injection of Wnt-1 mRNA into *Xenopus* embryos results in duplication of the dorsal axis and can easily be scored by direct inspection. Co-injection of Wnt-1 and Frzb resulted in the complete inhibition of secondary axis formation due to blockade of Wnt signaling (Fig. 7). Removal of the entire frizzled domain abolished the inhibitory activity of Frzb. Substantial inhibition of Wnt-1-mediated axis duplication was also observed when the CRD only was co-injected with Wnt-1. The C-terminal domain plays a role in this effect, as inhibition was more efficient in the presence of this domain. This suggests a possible role of the C-terminal domain in the stabilization of its tertiary structure affecting the binding affinity to Wnts, a possible involvement in Frzb turnover and increased solubility of the protein. In contrast to the co-immunoprecipitation data, no inhibition was observed in this *in vivo* assay with any of the deletion constructs affecting the CRD domain.

Wnt-5A was also co-injected with Frzb in *Xenopus* oocytes. Surprisingly, although Frzb binds Wnt-5A, no inhibition of Wnt-5A activity was observed. Co-injection of Wnt-5A and Frzb actually resulted in more pronounced changes in embryo phenotype. Thus, Frzb and related proteins are not always inhibitory and can be considered modulators of Wnt activity. Frzb may restrict Wnt activities and strictly regulate boundaries in certain systems by immobilizing Wnts in the cell, to the cell membrane or the pericellular matrix, while in other systems Frzb may function as a soluble factor enhancing Wnt secretion and even providing transportation to neighboring cells.

Example 20**Treatment of Deep Knee Defects with Frzb**

A young patient having a large defect in the articular surface of the knee joint is identified. A periosteal flap is obtained from the bone beneath the joint surface of rib cartilage according to standard surgical procedures.

- 5 The tissue flap is pre-incubated in a solution containing recombinant human, bovine or *Xenopus* Frzb protein. The Frzb-treated periosteal flap is then attached over the lesion in the articular surface of the knee joint by a sewing procedure using, for example, resolvable material. The joint is then closed and injected with a solution containing bovine, human or *Xenopus* Frzb protein in a pharmaceutically acceptable carrier. Injections are administered until cartilage repair is complete. The patient notices markedly less joint pain as the cartilage repair process progresses.
- 10 Examination by arthroscopy indicates repair of the lesion within several weeks following the initial procedure.

It is also contemplated that gene therapy protocols based on expression of Frzb cDNAs or genomic constructs can be used to facilitate *in vivo* cartilage, bone, muscle and nerve repair. Therapy may be achieved by genetically altering synoviocytes, periosteal cells, chondrocytes, myoblasts, osteoblasts or neural cells by transfection or infection with recombinant constructs directing expression of Frzb. Such altered cells can then be returned to the appropriate *in vivo* location. Gene transfer can be performed using numerous vectors well known in the art, including retroviruses, adenoviruses, herpesviruses and adeno-associated viruses.

Both *in vivo* and *ex vivo* approaches are anticipated for continuous delivery of Frzb for treating neuro-, myo-, osteo- and chondrodegenerative disorders. In addition, inducible promoter constructs may be employed in gene therapy applications of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES

(ii) TITLE OF THE INVENTION: ISOLATION AND USE OF TISSUE GROWTH-INDUCING FRZB PROTEIN

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

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- (A) APPLICATION NUMBER: 08/729,452

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(A) TELEPHONE: 619-235-8550

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2374 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 256...1230

(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAAGCTGTGA GATTGCCGGG GGAGGAGAAG CTCCCATATC ATTGTGTCCA CTCCAGGGC   180
GGGGAGGAGG AAACGGCGGA GCGGGCCTCT CGGCGTTCTC CGCACTGCTG CACCCTGCCC   240
CATCCTGCCG AGATC ATG GTC TGC GGG AGC CGA GGC GGG ATG CTG CTG CTG   291
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Met Val Cys Gly Ser Arg Gly Gly Met Leu Leu Leu

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Pro Ala Gly Leu Leu Ala Leu Ala Ala Leu Cys Leu Leu Arg Val Pro			
15	20	25	
GGA GCG CGG GCG GCC GCC TGT GAG CCC GTT CGC ATT CCC CTG TGC AAG			387
Gly Ala Arg Ala Ala Ala Cys Glu Pro Val Arg Ile Pro Leu Cys Lys			
30	35	40	
TCC CTG CCC TGG AAC ATG ACT AAG ATG CCC AAC CAC CTG CAC CAC AGC			435
Ser Leu Pro Trp Asn Met Thr Lys Met Pro Asn His Leu His His Ser			
45	50	55	60
ACC CAG GCC AAC GCC ATC CTG GCC ATC GAG CAG TTC GAA GGT CTG CTG			483
Thr Gln Ala Asn Ala Ile Leu Ala Ile Glu Gln Phe Glu Gly Leu Leu			
65	70	75	
GGC ACC CAC TGC AGC CCG GAT CTG CTC TTC TTC CTC TGT GCT ATG TAC			531
Gly Thr His Cys Ser Pro Asp Leu Phe Phe Leu Cys Ala Met Tyr			
80	85	90	
GCG CCC ATC TGC ACC ATT GAC TTC CAG CAC GAG CCC ATC AAG CCC TGC			579
Ala Pro Ile Cys Thr Ile Asp Phe Gln His Glu Pro Ile Lys Pro Cys			
95	100	105	
AAG TCT GTG TGC GAG CGG GCC CGG CAG GGC TGT GAG CCC ATC CTC ATC			627
Lys Ser Val Cys Glu Arg Ala Arg Gln Gly Cys Glu Pro Ile Leu Ile			
110	115	120	
AAG TAC CGC CAC TCG TGG CCG GAA AGC CTG GCC TGC GAG GAG CTG CCA			675
Lys Tyr Arg His Ser Trp Pro Glu Ser Leu Ala Cys Glu Glu Leu Pro			
125	130	135	140
GTA TAT GAC CGC GGC GTG TGC ATC TCT CCG GAG GCC ATC GTC ACT GCC			723
Val Tyr Asp Arg Gly Val Cys Ile Ser Pro Glu Ala Ile Val Thr Ala			

145	150	155	
GAC GGA GCC GAT TTT CCT ATG GAT TCC AGT AAT GGA AAC TGT AGA GGA			771
Asp Gly Ala Asp Phe Pro Met Asp Ser Ser Asn Gly Asn Cys Arg Gly			
160	165	170	
GCA AGC AGT GAA CGC TGC AAA TGT AAA CCA GTC AGA GCT ACA CAG AAG			819
Ala Ser Ser Glu Arg Cys Lys Cys Lys Pro Val Arg Ala Thr Gln Lys			
175	180	185	
ACC TAT TTC CGA AAC AAT TAC AAC TAT GTC ATT CGG GCT AAA GTT AAA			867
Thr Tyr Phe Arg Asn Asn Tyr Asn Tyr Val Ile Arg Ala Lys Val Lys			
190	195	200	
GAA ATA AAG ACC AAG TGT CAT GAT GTG ACT GCA GTA GTG GAG GTG AAG			915
Glu Ile Lys Thr Lys Cys His Asp Val Thr Ala Val Val Glu Val Lys			
205	210	215	220
GAG ATT TTA AAG GCT TCT CTG GTA AAC ATT CCA AGG GAA ACT GTG AAC			963
Glu Ile Leu Lys Ala Ser Leu Val Asn Ile Pro Arg Glu Thr Val Asn			
225	230	235	
CTT TAT ACC AGC TCT GGC TGC CTG TGT CCT CCA CTT AAC GTT AAT GAG			1011
Leu Tyr Thr Ser Ser Gly Cys Leu Cys Pro Pro Leu Asn Val Asn Glu			
240	245	250	
GAG TAT CTC ATC ATG GGC TAC GAA GAT GAA GAG CGC TCC AGA TTA CTG			1059
Glu Tyr Leu Ile Met Gly Tyr Glu Asp Glu Glu Arg Ser Arg Leu Leu			
255	260	265	
TTG GTA GAA GGT TCT ATT GCT GAG AAA TGG AAG GAT CGA CTT GGT AAA			1107
Leu Val Glu Gly Ser Ile Ala Glu Lys Trp Lys Asp Arg Leu Gly Lys			
270	275	280	
AAA GTT AAG CGG TGG GAT ATG AAG CTC CGT CAT CTT GGA CTG AAT ACA			1155
Lys Val Lys Arg Trp Asp Met Lys Leu Arg His Leu Gly Leu Asn Thr			

285 290 295 300
 AGT GAT TCT AGC CAT AGT GAT TCC ACT CAG AGT CAG AAG CCT GGC AGG 1203
 Ser Asp Ser Ser His Ser Asp Ser Thr Gln Ser Gln Lys Pro Gly Arg
 305 310 315
 AAT TCT AAC TCC CGG CAA GCA CGC AAC TAAATCCTGA AATGCAGAAA ATCCTCA 1257
 Asn Ser Asn Ser Arg Gln Ala Arg Asn
 320 325
 GTGGACTTCC TATTAAGACT TGCATTGCTG GACTAGCAAA GGCAAATTGC ACTATTGCAC 1317
 GTCATAGTCT ATTTTTTAGC CACAAAAATC AGGTGGTAAC TGATATTACT TCTATTTTTT 1377
 CTTTTGTTTT CTGCTTTTCT CTTTCCCCCA TTCCCTTTTT TGTGGTCTGA GTACAGATCC 1437
 TTAAATATAT TATATGTATT CTATTTCACT AATCATGGGA AAAGTGTCT TTGCAATAAT 1497
 AATAAATTA ACATGTTGAT ACCAGGGCCT CTTTGCTGGA GTAAATGTTA ATTTGCTGTT 1557
 CTGCACCCAG ATTGGGAATG CAATATTGGA TGCAAAGAGA GATTTCTGGT ATACAGAGAA 1617
 AGCTAGATAG GCTGTAAAGC ATACTTTGCT GATCTAATTA CAGCCTCATT CTTGCATGCC 1677
 TTTTGGCATT CTCCTCACGC TTAGAAAGTT CTAAATGTTT ATAAAGGTAA AATGACAGTT 1737
 TGAAATCAAA TGCCAACAGG CAGAGCAATC AAGCACCAGG AAGCATTAT GAAGAAATGA 1797
 CACATGAGAT GAATTATTTG CAAGATTGGC AGGAAGCAAA ATAAATAGCA TTAGGAGCTG 1857
 GGGATAGAGC ATTTTGCTG ACTGAGAAGC ACAACTGAAG CTAGTAGCTG TTGGGGTGTT 1917
 AACAGCAGCA TTTTCTTTT GACGATACAT TTGTTTGTCT GTGAATATAT TGATCAGCAT 1977
 TAGAGCAGTG GATTGTGACC AGACATCAGG TGTATCAGC ATAGCTCTGT TTAATTTGCT 2037
 TCCTTTTAGA TGAACGCATT GGTGTCTTTT TTTTCTTCTT TTAATAATAA TCTCCCTTGC 2097
 TGCATTTGAC CAGGAAAAGA AAGCATATAT GCATGTGCAC CGGGCTGTTA TTTTAAAGAT 2157
 ATGTAGCTCT ATAAACGCT ATAGTCAAAA GATGGTAAAA TGTGCAAGAT TCTGGGTGTG 2217
 TGTATTAATG TGTGTGTGTC CGCATACACT CACACTCAAG CTGAAGTGAA CGACAGGCCT 2277
 GTGCACTGGC CTGCACTTTA TCATTTGGAT TTGTGCTGTT TAATGCTCAG TAAAATATGC 2337
 TTAATAAAAG GAAAAAAAAA AAAAAAAAAA AAAAAAA 2374

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 325 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Cys Gly Ser Arg Gly Gly Met Leu Leu Leu Pro Ala Gly Leu
1 5 10 15
Leu Ala Leu Ala Ala Leu Cys Leu Leu Arg Val Pro Gly Ala Arg Ala
20 25 30
Ala Ala Cys Glu Pro Val Arg Ile Pro Leu Cys Lys Ser Leu Pro Trp
35 40 45
Asn Met Thr Lys Met Pro Asn His Leu His His Ser Thr Gln Ala Asn
50 55 60
Ala Ile Leu Ala Ile Glu Gln Phe Glu Gly Leu Leu Gly Thr His Cys
65 70 75 80
Ser Pro Asp Leu Leu Phe Phe Leu Cys Ala Met Tyr Ala Pro Ile Cys
85 90 95
Thr Ile Asp Phe Gln His Glu Pro Ile Lys Pro Cys Lys Ser Val Cys
100 105 110
Glu Arg Ala Arg Gln Gly Cys Glu Pro Ile Leu Ile Lys Tyr Arg His
115 120 125
Ser Trp Pro Glu Ser Leu Ala Cys Glu Glu Leu Pro Val Tyr Asp Arg
130 135 140
Gly Val Cys Ile Ser Pro Glu Ala Ile Val Thr Ala Asp Gly Ala Asp
145 150 155 160
Phe Pro Met Asp Ser Ser Asn Gly Asn Cys Arg Gly Ala Ser Ser Glu
165 170 175
Arg Cys Lys Cys Lys Pro Val Arg Ala Thr Gln Lys Thr Tyr Phe Arg
180 185 190
Asn Asn Tyr Asn Tyr Val Ile Arg Ala Lys Val Lys Glu Ile Lys Thr
195 200 205
Lys Cys His Asp Val Thr Ala Val Val Glu Val Lys Glu Ile Leu Lys
210 215 220
Ala Ser Leu Val Asn Ile Pro Arg Glu Thr Val Asn Leu Tyr Thr Ser

225	230	235	240
Ser Gly Cys Leu Cys Pro Pro Leu Asn Val Asn Glu Glu Tyr Leu Ile			
245	250	255	
Met Gly Tyr Glu Asp Glu Glu Arg Ser Arg Leu Leu Leu Val Glu Gly			
260	265	270	
Ser Ile Ala Glu Lys Trp Lys Asp Arg Leu Gly Lys Lys Val Lys Arg			
275	280	285	
Trp Asp Met Lys Leu Arg His Leu Gly Leu Asn Thr Ser Asp Ser Ser			
290	295	300	
His Ser Asp Ser Thr Gln Ser Gln Lys Pro Gly Arg Asn Ser Asn Ser			
305	310	315	320
Arg Gln Ala Arg Asn			
325			

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1484 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
(B) LOCATION: 208...1182
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGGGCCTGG GCGGSAGGGG CGGTGGCTGG AGCTCGGTAA AGCTCGTGGG ACCCCATTGG 60
GGGAATTTGA TCCAAGGAAG CGGTGATTGC CGGGGGAAGGA GAAGCTCCCA GATCCTTGTG 120
TCCACTTGCA GCGGGGGAGG CGGAGACGCG GAGCGGGCCT TTTGGCGTCC ACTGCGCGGC 180
TGCACCCTGC CCCATCCTGC CGGGATC ATG GTC TGC GGC AGC CCG GGA GGG ATG 234

Met Val Cys Gly Ser Pro Gly Gly Met

1 5

CTG CTG CTG CGG GCC GGG CTG CTT GCC CTG GCT GCT CTC TGC CTG CTC 282

Leu Leu Leu Arg Ala Gly Leu Leu Ala Leu Ala Ala Leu Cys Leu Leu

10 15 20 25

CGG GTG CCC GGG GCT CGG GCT GCA GCC TGT GAG CCC GTC CGC ATC CCC 330

Arg Val Pro Gly Ala Arg Ala Ala Ala Cys Glu Pro Val Arg Ile Pro

30 35 40

CTG TGC AAG TCC CTG CCC TGG AAC ATG ACT AAG ATG CCC AAC CAC CTG 378

Leu Cys Lys Ser Leu Pro Trp Asn Met Thr Lys Met Pro Asn His Leu

45 50 55

CAC CAC AGC ACT CAG GCC AAC GCC ATC CTG GCC ATC GAG CAG TTC GAA 426

His His Ser Thr Gln Ala Asn Ala Ile Leu Ala Ile Glu Gln Phe Glu

60 65 70

GGT CTG CTG GGC ACC CAC TGC AGC CCC GAT CTG CTC TTC TTC CTC TGT 474

Gly Leu Leu Gly Thr His Cys Ser Pro Asp Leu Leu Phe Phe Leu Cys

75 80 85

GCC ATG TAC GCG CCC ATC TGC ACC ATT GAC TTC CAG CAC GAG CCC ATC 522

Ala Met Tyr Ala Pro Ile Cys Thr Ile Asp Phe Gln His Glu Pro Ile

90 95 100 105

AAG CCC TGT AAG TCT GTG TGC GAG CGG GCC CGG CAG GGC TGT GAG CCC 570

Lys Pro Cys Lys Ser Val Cys Glu Arg Ala Arg Gln Gly Cys Glu Pro

110 115 120

ATA CTC ATC AAG TAC CGC CAC TCG TGG CCG GAG AAC CTG GCC TGC GAG 618

Ile Leu Ile Lys Tyr Arg His Ser Trp Pro Glu Asn Leu Ala Cys Glu

125 130 135

GAG CTG CCA GTG TAC GAC AGG GGC GTG TGC ATC TCT CCC GAG GCC ATC 666

Glu Leu Pro Val Tyr Asp Arg Gly Val Cys Ile Ser Pro Glu Ala Ile

140	145	150	
GTT ACT GCG GAC GGA GCT GAT TTT CCT ATG GAT TCT AGT AAC GGA AAC 714			
Val Thr Ala Asp Gly Ala Asp Phe Pro Met Asp Ser Ser Asn Gly Asn			
155	160	165	
TGT AGA GGG GCA AGC AGT GAA CGC TGT AAA TGT AAG CCT ATT AGA GCT 762			
Cys Arg Gly Ala Ser Ser Glu Arg Cys Lys Cys Lys Pro Ile Arg Ala			
170	175	180	185
ACA CAG AAG ACC TAT TTC CGG AAC AAT TAC AAC TAT GTC ATT CGG GCT 810			
Thr Gln Lys Thr Tyr Phe Arg Asn Asn Tyr Asn Tyr Val Ile Arg Ala			
190	195	200	
AAA GTT AAA GAG ATA AAG ACT AAG TGC CAT GAT GTG ACT GCA GTA GTG 858			
Lys Val Lys Glu Ile Lys Thr Lys Cys His Asp Val Thr Ala Val Val			
205	210	215	
GAG GTG AAG GAG ATT CTA AAG TCC TCT CTG GTA AAC ATT CCA CGG GAC 906			
Glu Val Lys Glu Ile Leu Lys Ser Ser Leu Val Asn Ile Pro Arg Asp			
220	225	230	
ACT GTC AAC CTC TAT ACC AGC TCT GGC TGC CTC TGC CCT CCA CTT AAT 954			
Thr Val Asn Leu Tyr Thr Ser Ser Gly Cys Leu Cys Pro Pro Leu Asn			
235	240	245	
GTT AAT GAG GAA TAT ATC ATC ATG GGC TAT GAA GAT GAG GAA CGT TCC 1002			
Val Asn Glu Glu Tyr Ile Ile Met Gly Tyr Glu Asp Glu Glu Arg Ser			
250	255	260	265
AGA TTA CTC TTG GTG GAA GGC TCT ATA GCT GAG AAG TGG AAG GAT CGA 1050			
Arg Leu Leu Leu Val Glu Gly Ser Ile Ala Glu Lys Trp Lys Asp Arg			
270	275	280	
CTC GGT AAA AAA GTT AAG CGC TGG GAT ATG AAG CTT CGT CAT CTT GGA 1098			
Leu Gly Lys Lys Val Lys Arg Trp Asp Met Lys Leu Arg His Leu Gly			

285 290 295

CTC AGT AAA AGT GAT TCT AGC AAT AGT GAT TCC ACT CAG AGT CAG AAG 1146
Leu Ser Lys Ser Asp Ser Ser Asn Ser Asp Ser Thr Gln Ser Gln Lys

300 305 310

TCT GGC AGG AAC TCG AAC CCC CGG CAA GCA CGC AAC TAAATCCCGA AATACA 1198
Ser Gly Arg Asn Ser Asn Pro Arg Gln Ala Arg Asn

315 320 325

AAAAGTAACA CAGTGGACTT CCTATTAAGA CTTACTTGCA TTGCTGGACT AGCAAAGGAA 1258
AATTGCACTA TTGCACATCA TATTCTATTG TTTACTATAA AAATCATGTG ATAAGTGATT 1318
ATTACTTCTG TTTCTCTTTT GGTTCCTGCT TCTCTCTTCT CTCAACCCCT TTGTAATGGT 1378
TTGGGGGCAG ACTCTTAAGT ATATTGTGAG TTTTCTATTT CACTAATCAT GAGAAAAACT 1438
GTTCTTTTGC AATAATAATA AATTAAACAT GCTGTAAAAA AAAAAA 1484

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 325 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Cys Gly Ser Pro Gly Gly Met Leu Leu Leu Arg Ala Gly Leu
1 5 10 15
Leu Ala Leu Ala Ala Leu Cys Leu Leu Arg Val Pro Gly Ala Arg Ala
20 25 30
Ala Ala Cys Glu Pro Val Arg Ile Pro Leu Cys Lys Ser Leu Pro Trp
35 40 45
Asn Met Thr Lys Met Pro Asn His Leu His His Ser Thr Gln Ala Asn

50 55 60
 Ala Ile Leu Ala Ile Glu Gln Phe Glu Gly Leu Leu Gly Thr His Cys
 65 70 75 80
 Ser Pro Asp Leu Leu Phe Phe Leu Cys Ala Met Tyr Ala Pro Ile Cys
 85 90 95
 Thr Ile Asp Phe Gln His Glu Pro Ile Lys Pro Cys Lys Ser Val Cys
 100 105 110
 Glu Arg Ala Arg Gln Gly Cys Glu Pro Ile Leu Ile Lys Tyr Arg His
 115 120 125
 Ser Trp Pro Glu Asn Leu Ala Cys Glu Glu Leu Pro Val Tyr Asp Arg
 130 135 140
 Gly Val Cys Ile Ser Pro Glu Ala Ile Val Thr Ala Asp Gly Ala Asp
 145 150 155 160
 Phe Pro Met Asp Ser Ser Asn Gly Asn Cys Arg Gly Ala Ser Ser Glu
 165 170 175
 Arg Cys Lys Cys Lys Pro Ile Arg Ala Thr Gln Lys Thr Tyr Phe Arg
 180 185 190
 Asn Asn Tyr Asn Tyr Val Ile Arg Ala Lys Val Lys Glu Ile Lys Thr
 195 200 205
 Lys Cys His Asp Val Thr Ala Val Val Glu Val Lys Glu Ile Leu Lys
 210 215 220
 Ser Ser Leu Val Asn Ile Pro Arg Asp Thr Val Asn Leu Tyr Thr Ser
 225 230 235 240
 Ser Gly Cys Leu Cys Pro Pro Leu Asn Val Asn Glu Glu Tyr Ile Ile
 245 250 255
 Met Gly Tyr Glu Asp Glu Glu Arg Ser Arg Leu Leu Leu Val Glu Gly
 260 265 270
 Ser Ile Ala Glu Lys Trp Lys Asp Arg Leu Gly Lys Lys Val Lys Arg
 275 280 285
 Trp Asp Met Lys Leu Arg His Leu Gly Leu Ser Lys Ser Asp Ser Ser
 290 295 300
 Asn Ser Asp Ser Thr Gln Ser Gln Lys Ser Gly Arg Asn Ser Asn Pro
 305 310 315 320
 Arg Gln Ala Arg Asn
 325

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 111 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Gln Pro Ile Ser Ile Pro Leu Cys Thr Asp Ile Ala Tyr Asn Gln
 1 5 10 15
 Thr Ile Met Pro Asn Leu Leu Gly His Thr Asn Gln Glu Asp Ala Gly
 20 25 30
 Leu Glu Val His Gln Phe Tyr Pro Leu Val Lys Val Gln Cys Ser Ala
 35 40 45
 Glu Leu Lys Phe Phe Leu Cys Ser Met Tyr Ala Pro Val Cys Thr Val
 50 55 60
 Leu Glu Gln Ala Leu Pro Pro Cys Arg Ser Leu Cys Glu Arg Ala Gln
 65 70 75 80
 Gly Cys Glu Ala Leu Met Asn Lys Phe Gly Phe Gln Trp Pro Asp Thr
 85 90 95
 Leu Lys Cys Glu Lys Phe Pro Val His Gly Arg Gly Glu Leu Cys
 100 105 110

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 111 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Cys Glu Pro Ile Thr Ile Ser Ile Cys Lys Asn Ile Pro Tyr Asn Met
 1 5 10 15
 Thr Ile Met Pro Asn Leu Ile Gly His Thr Lys Gln Glu Glu Ala Gly
 20 25 30
 Leu Glu Val His Gln Phe Ala Pro Leu Val Lys Ile Gly Cys Ser Asp
 35 40 45
 Asp Leu Gln Leu Phe Leu Cys Ser Leu Tyr Val Pro Val Cys Thr Ile
 50 55 60
 Leu Glu Arg Pro Ile Pro Pro Cys Arg Ser Leu Cys Glu Ser Ala Arg
 65 70 75 80
 Val Cys Glu Lys Leu Met Lys Thr Tyr Asn Phe Asn Trp Pro Glu Asn
 85 90 95
 Leu Glu Cys Ser Lys Phe Pro Val His Gly Gly Glu Asp Leu Cys
 100 105 110

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 319 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ser Pro Thr Arg Lys Leu Asp Ser Phe Leu Leu Leu Val Ile Pro
 1 5 10 15
 Gly Leu Val Leu Leu Leu Leu Pro Asn Ala Tyr Cys Ala Ser Cys Glu
 20 25 30
 Pro Val Arg Ile Pro Met Cys Lys Ser Met Pro Trp Asn Met Thr Lys
 35 40 45
 Met Pro Asn His Leu His His Ser Thr Gln Ala Asn Ala Ile Leu Ala
 50 55 60

Ile Glu Gln Phe Glu Gly Leu Leu Thr Thr Glu Cys Ser Gln Asp Leu
 65 70 75 80
 Leu Phe Phe Leu Cys Ala Met Tyr Ala Pro Ile Cys Thr Ile Asp Phe
 85 90 95
 Gln His Glu Pro Ile Lys Pro Cys Lys Ser Val Cys Glu Arg Ala Arg
 100 105 110
 Ala Gly Cys Glu Pro Ile Leu Ile Lys Tyr Arg His Ile Trp Pro Glu
 115 120 125
 Ser Leu Ala Cys Glu Glu Leu Pro Val Tyr Asp Arg Gly Val Cys Ile
 130 135 140
 Ser Pro Glu Ala Ile Val Thr Val Glu Gln Gly Thr Asp Ser Met Pro
 145 150 155 160
 Asp Phe Pro Met Asp Ser Asn Asn Gly Asn Cys Gly Ser Thr Ala Gly
 165 170 175
 Glu His Cys Lys Cys Lys Pro Met Lys Ala Ser Gln Lys Thr Tyr Leu
 180 185 190
 Lys Asn Asn Tyr Asn Tyr Val Ile Arg Ala Lys Val Lys Glu Val Lys
 195 200 205
 Val Lys Cys His Asp Ala Thr Ala Ile Val Glu Val Lys Glu Ile Leu
 210 215 220
 Lys Ser Ser Leu Val Asn Ile Pro Lys Asp Thr Val Ile Leu Tyr Thr
 225 230 235 240
 Asn Ser Gly Cys Leu Cys Pro Gln Leu Val Ala Asn Glu Glu Tyr Ile
 245 250 255
 Ile Met Gly Tyr Glu Asp Lys Glu Arg Thr Arg Leu Leu Leu Val Glu
 260 265 270
 Gly Ser Leu Ala Glu Lys Trp Arg Asp Arg Leu Ala Lys Lys Val Lys
 275 280 285
 Arg Trp Asp Gln Lys Leu Arg Arg Pro Arg Lys Ser Lys Asp Pro Val
 290 295 300
 Ala Pro Ile Pro Asn Lys Asn Ser Asn Ser Arg Gln Ala Arg Ser
 305 310 315

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 318 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Val Cys Gly Ser Gly Gly Met Leu Leu Leu Ala Gly Leu Leu Ala
 1 5 10 15
 Leu Ala Ala Leu Leu Leu Arg Val Pro Gly Ala Arg Ala Ala Ala Cys
 20 25 30
 Glu Pro Val Arg Ile Pro Leu Cys Lys Ser Leu Pro Trp Asn Met Thr
 35 40 45
 Lys Met Pro Asn His Leu His His Ser Thr Gln Ala Asn Ala Ile Leu
 50 55 60
 Ala Ile Glu Gln Phe Glu Gly Leu Leu Gly Thr His Cys Ser Pro Asp
 65 70 75 80
 Leu Leu Phe Phe Leu Cys Ala Met Tyr Ala Pro Ile Cys Thr Ile Asp
 85 90 95
 Phe Gln His Glu Pro Ile Lys Pro Cys Lys Ser Val Cys Glu Arg Ala
 100 105 110
 Arg Gln Gly Cys Glu Pro Ile Leu Ile Lys Tyr Arg His Ser Trp Pro
 115 120 125
 Glu Ser Leu Ala Cys Glu Glu Leu Pro Val Tyr Asp Arg Gly Val Cys
 130 135 140
 Ile Ser Pro Glu Ala Ile Val Thr Ala Asp Gly Ala Asp Phe Pro Met
 145 150 155 160
 Asp Ser Ser Asn Gly Asn Cys Arg Gly Ala Ser Ser Glu Arg Cys Lys
 165 170 175
 Cys Lys Pro Arg Ala Ile Gln Lys Thr Tyr Phe Arg Asn Asn Tyr Asn
 180 185 190
 Tyr Val Ile Arg Ala Lys Val Lys Glu Ile Lys Ile Lys Cys His Asp
 195 200 205
 Val Thr Ala Val Val Glu Val Lys Glu Ile Leu Lys Ser Ser Leu Val
 210 215 220

Asn Ile Pro Arg Asp Thr Val Asn Leu Tyr Thr Ser Ser Gly Cys Leu
 225 230 235 240
 Cys Pro Pro Leu Asn Val Asn Glu Glu Tyr Ile Ile Met Gly Tyr Glu
 245 250 255
 Asp Glu Glu Arg Ser Arg Leu Leu Leu Val Glu Gly Ser Ile Ala Glu
 260 265 270
 Lys Trp Lys Asp Arg Leu Gly Lys Lys Val Lys Arg Trp Asp Met Lys
 275 280 285
 Leu Arg His Leu Gly Leu Ser Asp Ser Ser Ser Asp Ser Thr Gln Ser
 290 295 300
 Gln Lys Pro Gly Arg Asn Ser Asn Ser Arg Gln Ala Arg Asn
 305 310 315

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Thr Val Asn Leu Tyr Thr Ser Ala Gly Cys Leu Cys Pro Pro Leu
 1 5 10 15
 Asn Val Asn Glu Glu Tyr Leu Ile Met Gly Tyr Glu Phe Pro
 20 25 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GARACHGTSA AYCTBTAYAC N

21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

RAAYTCRTAN CCCATNAT

18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Other

(B) LOCATION: 13...13

(D) OTHER INFORMATION: Aspartic Acid or Histidin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Val Cys Ile Ser Pro Glu Ala Ile Val Thr Ala Xaa Gly Ala Asp
1 5 10 15
Phe Pro Met

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gln Gly Cys Glu Pro Ile Leu Ile Lys
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gln Gly Cys Glu Pro Ile Leu Ile Cys Ala Trp Pro Pro Leu Tyr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Glu Thr Val Asn Leu Tyr Thr Ser Ala Gly Cys Leu Cys Pro Pro Leu
1 5 10 15
Asn Val Asn Glu Glu Tyr Leu Ile Met Gly Tyr Glu
 20 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Glu Thr Val Asn Leu Tyr Thr Ser Ser Gly Cys Leu Cys Pro Pro Leu
1 5 10 15
Asn Val Asn Glu Glu Tyr Leu Ile Met Gly Tyr Glu
 20 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTCTGGCTG CCTGTGTCCT CCACTTAACG

30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCTCCACTTA ACGTTAATGA GGAGTATCTC

30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGGAACATGA CTAAGATGCC C

21

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CATATACTGG CAGCTCCTCG

20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTCTTTTGGG AAGCCTTCAT GG

22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCATCGTGGC ATTTCACTTT CA

22

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1291 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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TTTACTGTGC CAGTCTTCCC TGTAACCAGC GACCTGTATT CCCCCAAGTA AGCCTACACA   60
TACAGGTTGG GCAGAATAAC AATGTCTCCA ACAAGGAAAT TGGACTCATT CCTGCTACTG   120
GTCATACCTG GACTGGTGCT TCTCTTATTA CCAATGCTT ACTGTGCTTC GTGTGAGCCT   180
GTGCGGATTC CCATGTGCAA ATCTATGCCA TGAACATGA CCAAGATGCC CAACCATCTC   240
CACCACAGCA CTCAAGCCAA TGCTATCCTG GCAATTGAAC AGTTTGAAGG TTGCTGACC   300
ACTGAATGTA GCCAGGACCT TTTGTTCTTT CTGTGTGCCA TGTATGCCCC CATTTGTACC   360
ATCGATTTCC AGCATGAACC AATTAAGCCT TGCAAGTCCG TGTGCGAAAG GGCCAGGGCC   420
GGCTGTGAGC CCATTCTCAT AAAGTACCGG CACACTTGGC CAGAGAGCCT GGCATGTGAA   480
GAGCTGCCCC TATATGACAG AGGAGTCTGC ATCTCCCCAG AGGCTATCGT CACAGTGGAA   540
CAAGGAACAG ATTCAATGCC AGACTTCCCC ATGGATTCAA ACAATGGAAG TTGCGGAAGC   600
ACGGCAGGTG AGCACTGTAA ATGCAAGCCC ATGAAGGCTT CCCCCAAGAC GTATCTCAAG   660
AATAATTACA ATTATGTAAT CAGAGCAAAA GTGAAAGAGG TGAAAGTGAA ATGCCACGAC   720
GCAACAGCAA TTGTGGAAGT AAAGGAGATT CTCAAGTCTT CCCTAGTGAA CATTCTTAAA   780
GACACAGTGA CACTGTACAC CAACTCAGGC TGCTTGCGCC CCCAGCTTGT TGCCAATGAG   840
GAATACATAA TTATGGGCTA TGAAGACAAA GAGCGTACCA GGCTTCTACT AGTGGAAGGA   900
TCCTTGGCCG AAAAATGGAG AGATCGTCTT GCTAAGAAAG TCAAGCGCTG GGATCAAAAG   960
CTTCGACGTC CCAGGAAAAG CAAAGACCCC GTGGCTCAA TTCCCAACAA AAACAGCAAT  1020
TCCAGACAAG CGCGTAGTTA GACTAACGGA AAGGTGTATG GAACTCTAT GGACTTTGAA  1080
ACTAAGATTT GCATTGTTGG AAGAGCAAAA AAGAAATTGC ACTACAGCAC GTTATATTCT  1140
TTGTTTACT ACAAGAAGCT GGTTAGTTG ATTGTAGTTC TCCTTTCCTT CTTTTTTTAA  1200
TAACTATATT GCACGTGTTT CAGGCAGTTT ATCAACTTCC AGTGACAGAG CAGTGACTGA  1260
ATGTAGCTAA GAGCCTATCA TCTGATCACT A                                1291
```

WHAT IS CLAIMED IS:

1. An isolated polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1, 3 or 23.
2. An isolated polynucleotide encoding a native Frzb protein, said polynucleotide capable of hybridizing to a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1 at 55°C in 3 x SSC, 0.1% SDS.
- 5 3. An isolated Frzb protein encoded by the polynucleotide of Claim 2.
4. An isolated recombinant Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7.
5. The isolated Frzb protein of Claim 4, wherein at least one acidic amino acid contained therein is replaced with a different acidic amino acid.
- 10 6. The isolated Frzb protein of Claim 4, wherein at least one basic amino acid contained therein is replaced with a different basic amino acid.
7. The isolated Frzb protein of Claim 4, wherein at least one nonpolar amino acid contained therein is replaced with a different nonpolar amino acid.
8. The isolated Frzb protein of Claim 4, wherein at least one uncharged polar amino acid contained
- 15 therein is replaced with a different uncharged polar amino acid.
9. The isolated Frzb protein of Claim 4, wherein at least one aromatic amino acid contained therein is replaced with a different aromatic amino acid.
10. The protein having the amino acid sequence shown in the SEQ ID NO: 2 of Claim 4, wherein said protein is obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 1.
- 20 11. The protein having the amino acid sequence shown in the SEQ ID NO: 4 of Claim 4, wherein said protein is obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 3.
12. The protein having the amino acid sequence shown in the SEQ ID NO: 7 of Claim 3, wherein said protein is obtained by expression of a polynucleotide having the sequence shown in SEQ ID NO: 23.
13. Isolated mammalian Frzb protein having a molecular weight of about 36 kilodaltons.
- 25 14. A pharmaceutical composition for inducing cartilage, bone, nerve or muscle growth comprising the isolated Frzb protein of Claim 3, or a Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7, in a pharmaceutically acceptable carrier.
15. The composition of Claim 6, wherein said carrier comprises fibrin glue, freeze-dried cartilage grafts or collagen.
- 30 16. The composition of Claim 7, wherein said composition further comprises cartilage progenitor cells, chondroblasts or chondrocytes.
17. The composition of Claim 6, wherein said Frzb protein is coated onto or mixed with a resorbable or nonresorbable matrix.
18. The composition of Claim 6, wherein said Frzb protein is mixed with a biodegradable polymer.

19. A method of treating a cartilage, bone, nerve or muscle growth disorder in a mammal in need thereof, comprising the step of administering to said mammal an effective cartilage, bone, nerve or muscle-inducing amount of the pharmaceutical composition of Claim 6 at the site of said disorder.

20. The method of Claim 19, wherein said disorder is selected from the group consisting of subglottic stenosis, tracheomalacia, chondromalacia patellae, osteoarthritis, joint surface lesions, neurodegenerative disorders, myodegenerative disorders and osteodegenerative disorders.

21. The method of Claim 19, wherein said administering step is intravenous, intrathecal, intracranial, intramuscular or subcutaneous.

22. The method of Claim 19, wherein said mammal is a human.

10 23. A method of stimulating cartilage formation in a mammal, comprising the steps of:
combining the isolated Frzb protein of Claim 3, or a Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7, with a matrix to produce a product that facilitates administration of said protein; and

15 implanting said product into the body of a mammal to stimulate cartilage formation at the site of implantation.

24. The method of Claim 23, wherein said matrix comprises a cellular material.

25. The method of Claim 23, wherein said combining step additionally comprises mixing of viable chondroblasts or chondrocytes.

26. The method of Claim 23, wherein said implanting is subcutaneous or intramuscular.

20 27. The method of Claim 23, wherein said mammal is a human.

28. A method of modulating Wnt-mediated signaling in a cell, comprising contacting said cell with an effective Wnt-modulating amount of the isolated Frzb protein of Claim 3, a Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7, or a Wnt-modulating fragment thereof.

29. The method of Claim 28, wherein said cell is contacted *in vivo*.

25 30. The method of Claim 28, wherein said Wnt is selected from the group consisting of Wnt-8, Wnt-1, Wnt-2, Wnt-3, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A and Wnt-7B.

31. A method of modulating Wnt-mediated signaling in a cell, comprising contacting said cell with a recombinant construct comprising the coding region of SEQ ID NO: 1, 3 or 23, or a region encoding an active Wnt-modulating fragment thereof, operably linked to a heterologous promoter in an expression vector.

30 32. The method of Claim 31, wherein said Wnt is selected from the group consisting of Wnt-8, Wnt-1, Wnt-2, Wnt-3, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A and Wnt-7B.

33. A method of inhibiting the growth of a Wnt growth factor-expressing tumor in a mammal, comprising the step of contacting said tumor with an effective tumor growth-inhibiting amount of the isolated Frzb protein of Claim 3, or a Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7.

34. The method of Claim 33, wherein said tumor is a mammary or intestinal tumor.

5 35. The method of Claim 33, wherein said mammal is a human.

36. A method of inhibiting the growth of a Wnt growth factor-expressing tumor in a mammal, comprising the step of contacting said tumor with a recombinant construct comprising the coding region of SEQ ID NO: 1, 3 or 23 operably linked to a heterologous promoter in an expression vector.

37. The method of Claim 36, wherein said construct is injected into said tumor.

10 38. The method of Claim 36, wherein said recombinant construct is systemically administered to said mammal.

39. The method of Claim 36, wherein said expression vector is a plasmid vector, retroviral vector or adenoviral vector.

15 40. Isolated antibodies to Frzb protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 7.

41. The antibodies of Claim 40, wherein said antibodies are polyclonal.

42. The antibodies of Claim 40, wherein said antibodies are polyclonal.

43. A method of facilitating tissue growth or repair, comprising the steps of:
isolating cells from said tissue;
20 introducing a recombinant construct expressing Frzb into said cells; and
returning said cells to said tissue.

44. The method of Claim 43, wherein said recombinant construct comprises a retroviral vector, adenoviral vector, herpesvirus vector or adeno-associated viral vector.

25 45. The method of Claim 43, wherein said tissue is selected from the group consisting of cartilage, muscle, bone and neural tissue.

46. A method of identifying a compound which affects Frzb activity, comprising:
contacting isolated Frzb with said compound; and
determining Frzb activity, wherein an increase in activity compared to Frzb alone indicates that
said compound is a Frzb activator and a decrease in activity indicates that said compound is a Frzb
30 inhibitor.

47. The method of Claim 46, wherein said determining step comprises an *in vivo* chondrogenesis assay.

48. An isolated Frzb-derived peptide having an amino acid sequence shown in a SEQ ID NO: selected from the group consisting of SEQ ID NO: 12, 13, 14, 15 and 16.

49. A recombinant construct comprising the coding region of SEQ ID NO: 1, 3 or 23 operably linked
35 to a heterologous promoter in an expression vector.

50. The recombinant construct of Claim 49, wherein said expression vector is eukaryotic.

51. The recombinant construct of Claim 49, wherein said expression vector is prokaryotic.
52. The recombinant construct of Claim 49, wherein said expression vector is pcDNA3.
53. A cultured mammalian cell line containing the recombinant construct of Claim 49.
54. An isolated recombinant Frzb protein containing amino acids 33-319 of SEQ ID NO: 7, 33-325 of
5 SEQ ID NO: 2 or 33-325 of SEQ ID NO: 4.

AATAGATGCC GCGGCCCCAG AAGTCTTAGA CGTCGGGAAA GAGCAGCCGG AGAGGCCGGG CTGGCGCTCG GCGAGCTTTT TGGACCCCA TTGAGGGAAT TgATCCAA
 GAAGTGTGA GATTCCGGG GGAGGAGAG CTCCTATATC ATTGTGTCCA CTTCAGGGC GGGAGAGG AAACGGGGA GCGGCTCTT CGGCTTCTC GCACTGTCTG CACCTGTGCC
 CATCTGCCG AGATCATGTT CTGCGGGAG CGAGCGGGA TGCTGTGCT GCGGCCGGG CTACTCGCC CTGCTGCTC GCGTGCCTG GAGCGGGG GCGCGCTGT
 M V C G S R G M L L L P A G L L A L A A L C L L R V P G A R A A A C 35
 GAGCCGTTT GCATTCCCT GTGCAAGTCC CTGCCCTGGA ACATGACTAA GATGCCCAAC CACCTGCACC ACAGCACCA GGCCAAAGCC ATCTGGCCA TCGAGCAGTT CGAAGTCTG
 E P V R I P L C K S L P W N M T K M P N H L H S T Q A N A I L A I E Q F E G L 75
 CTGGCACCC ACTGCAGCCC GGATCTGCTC TTCTTCTCT GTGCTATGA CGCGCCCATC TGCAACATTG ACTTCCAGCA CGAGCCCATC AAGCCCTGCA AGTCTGTGTG CGAGCGGGC
 L G T H C S P D L L F F L C A M Y A P I C T I D F Q H E P I K P C K S V C E R A 115
 CGGAGGGCT GTGAGCCCAT CCTCATCAAG TACCGCCACT CGTGGCCGGA AAGCCTGGCC TCGAGGAGC TGCCAGTATA TGACCGCGGC GTGTGCACT CTCCGGAGGC CATCTGCTACT
 R Q G C E P I L I K Y R H S W P E S L A C E E L P V Y D R G V C I S P E A I V T 155
 GCGACGGAG CCGATTTTCC TATGATTTCC AGTAATGGAA ACTGTAGAGG AGCAAGCAGT GAACGTGCA AATGTAAACC AGTCAGAGCT ACACAGAAGA CCTATTTCCG AAACAATTAC
 A D G A D F P M D S S N G N C R G A S S E R C K C K P V R A T Q K T Y F R N N Y 195
 AACTATGTCA TTCGGGCTAA AGTTAAAGAA ATAAAGACCA AGTGTCTATGA TGTGACTGCA GTAGTGGAGG TGAAGGAGAT TTTAAAGGCT TCTCTGGTAA ACATTCCAG GGAAGCTGTG
 N Y V I R A K V K E I K T K C H D V T A V V E V K E I L K A S L V N I P R E T V 235
 AACCTTTATA CCAGCTCTGG CTGCTGTGT CCTCCACTTA AGTTAATGA GGATATCTC ATCATGGCT ACAGAGATGA AGAGCGCTCC AGATTACTGT TGGTAGAAGG TTCTATTGCT
 N L Y I S S S L C P L N V N E E Y L I M G Y E D E E R S R L L L V E G S I A 275
 GAGAAATGA AGGATCGACT TGGTAAAGAA GTTAAGCGGT GGGATATGAA CGTCCGTCTAT CTGGAGTGA ATACAAGTGA TTCTAGCCAT AGTGATTCCA CTCAGAGTCA GAGCGCTGCG
 E K W K D R L G K K V K R W D M K L R H L G L N T S D S S H S D S T Q S Q K P G 315
 AGGAATCTA ACTCCCGCA AGCAGCGAAC TAAATCCTGA AATGCAGAAA ATCTCAGTG GACTTCTAT TAAGACTTGC ATTGCTGGAC TAGCAAGGC AAATTGCACT ATTGCACGTC
 R N S N S R Q A R N
 ATAGTCTATT TTTAGCCAC AAAATCAGG TGGTAACTGA TATTACTTCT ATTTTCTCT TGTCTTCTG CTCTTCTCT TCCTCGATTC CCTTTTGT GGTCTGAGTA CAGATCCCTA
 AATATATAT ATGTATTCTA TTCACTAAT CATGGGAAA CTGTTCTTTG CAATATATAT AATTAACA TGTGTATACC AGGCTCTCTT TCGTGGAGTA AATGTTAAT TGTGTTCTG
 CACCCAGAT GGGATGCAA TATTGGATGC AAAGAGAT TTCTGGTATA CAGAGAAAGC TAGATAGGCT GTAAAGCATA CTGTCTCAT CTAAATACAG CCTCATCTT GCATGCTCTT
 TGGCAITCTC CTCACGCTTA GAAGTTCTA AATGTTATA AAGTAAAT GACAGTTGA ATCAATGC CAACGGCAG AGCAATCAG CACAGGAAG CATTAAGAA GAATGACAC
 ATGAGATGAA TTATTGCAA GATTGGCAGG AAGCAAATA AATGACATTA TGAGCTGGG ATGAGCAAT TTGCTCTACT GAGAAGCACA ACTGAAGCTA GTAGCTGGTG GGGTGTAAAC
 AGCAGCAATT TTCTTTTGAC GATACATTTG TTTGTCTGTG AATATATTGA TCAGCATTAG AGCAGTGGT TGTGACCAGA CATCAGTGT TATCAGCATA GCTCTGTTTA ATTGCTTCC
 TTTTAGATG ACGATTTGGT GTCTTTTTT TCCTTTTTTA AATTAATCT CCCTGCTGCT ATTTGACCCAG GAAAGAAAG CATATATGCA TGTGACCGG GCTGTTTATT TTRAGATATG
 TAGCTCTATA AAACGCTATA GTCAAAAGAT GGTAAATCT GGAATCTCT ATTAATGTGT GTGTGCTCGG ATACACTCAC ACTCAAGCTG AACTGAAGCA CAGGCTGTG
 CACTGGCTG CACTTTATCA TTGGATTG TGCTGTTTAA TGTCTAGTAA AATATGCTTA ATAAAGGAA AAAAAAAA AAAAAAAA AAAA

FIG. 1

bovine	MVCGSR	<u>GGML LLPAGLLALAL ALCLLRV</u>	PGA	RAACEPVRI	PLCKSLPWNM	50
human	-----P-----	-----R-----	-----	-----	-----	50
bovine	TKMPNHLHHS	TQANAILAIE	<u>QFEGLLGTHC</u>	<u>SPDLLFFLCA</u>	MYAPICTIDF	100
human	-----	-----	-----	-----	-----	100
bovine	QHEPIKPCKS	VCERARQGCE	PILIKYRHSW	PESLACEELP	VYDRGVCISP	150
human	-----	-----	-----	--N-----	-----	150
bovine	EAIVTADGAD	FPMDSNGNC	RGASSERCKC	KPVRATQKTY	FRNNYNYVIR	200
human	-----	-----	-----	--I-----	-----	200
bovine	AKVKEIKTKC	HDVTAVVEVK	EILKASLVNI	PRETVNLYTS	SGCLCPPLNV	250
human	-----	-----	-----S-----	--D-----	-----	250
bovine	NEEYLIMGYE	DEERSRLLLV	EGSIAEKWKD	RLGKKVKRWD	MKLRHLGLNT	300
human	-----I-----	-----	-----	-----	-----SK	300
bovine	SDSSHSDSTQ	SQKPGRNSNS	RQARN			325
human	-----N-----	-----S-----	-----P-----	-----	-----	325

FIG. 2A

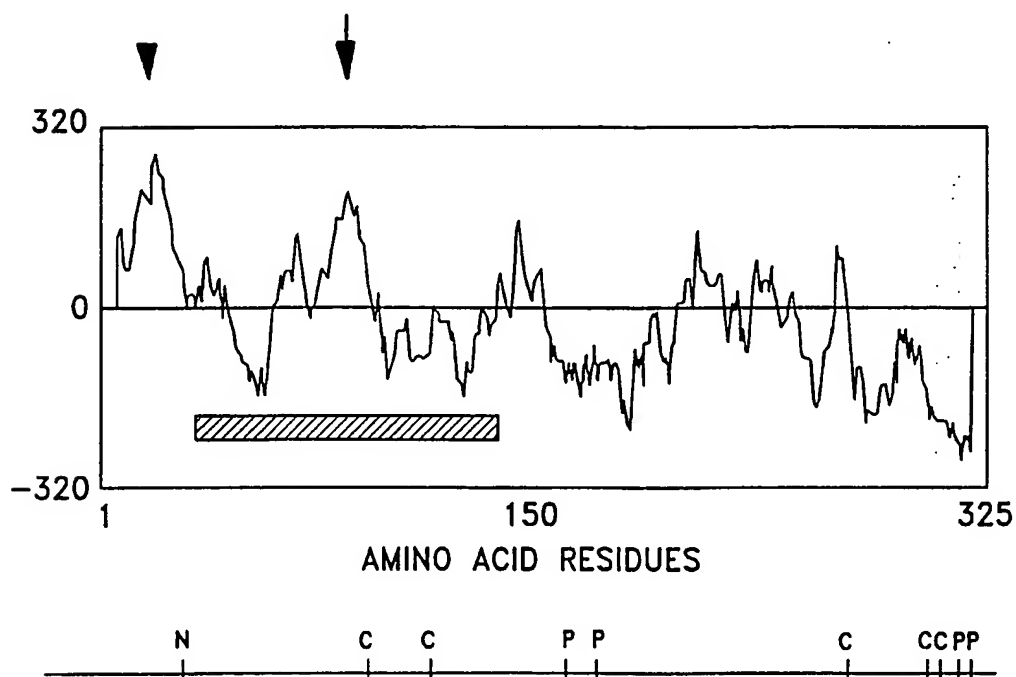


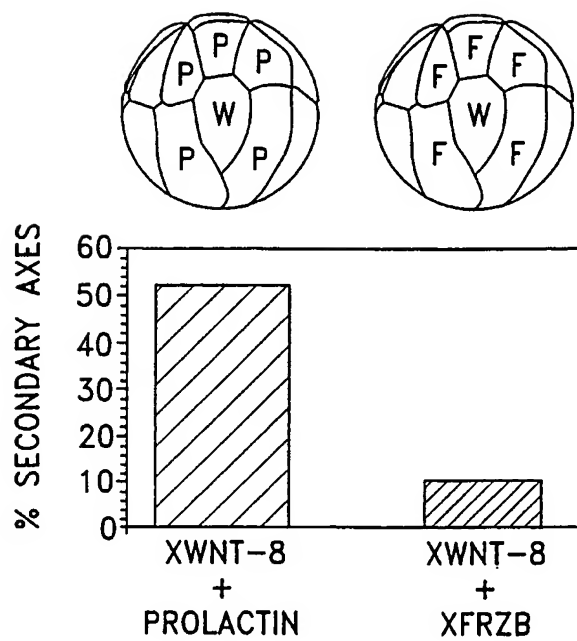
FIG. 2B

Rat fz-1	QCFISITPLOT	DIAYNQTUMP	NLIGHTNQED	AGLEVHQFYP	IVKVQCSAEN	160
Drosophila frizzled	CEPITISICK	NIPYNNMIMP	NLIGHITKQEE	AGLEVHQEAP	IVKIGCSDGL	102
bovine frzb	CEPVRITPCK	SLEWNTMKMP	NHLHSTQAN	ALLAIEQFEG	LLGTHCSPDL	84
human frzb	CEPVRITPCK	SLEWNTMKMP	NHLHSTQAN	ALLAIEQFEG	LLGTHCSPDL	84
Rat fz-1	KFFLCSTMAP	VCTVLEQALP	--PORSICER	A--QCEALMN	KFGFQWPDIL	207
Drosophila frizzled	QLFLCSLAMP	VCTTLERPPI	--PORSICER	AR-VCEKLMK	TYNFNWPENL	149
bovine frzb	LEFLCAMPAP	ICTIIDFQHEP	IKPKCKSVCEP	ARQGCCEPILI	KMRHSWPESL	134
human frzb	LEFLCAMPAP	ICTIIDFQHEP	IKPKCKSVCEP	ARQGCCEPILI	KMRHSWPENL	134
Rat fz-1	KCEKFPVHCR	CELC				221
Drosophila frizzled	ECSKFPVHGG	EDLC				163
bovine frzb	ACEELPVYDR	G-VC				147
human frzb	ACEELPVYDR	G-VC				147

FIG.3

xFrzb	MSPTKLDSP	YCSCEPVRI	PMCKSPWNM	46
bFrzb	MVCSRGGM	ALCLIRVEGA	PLCKSPWNM	50
hFRZB	MVCSPPGM	ALCLIRVEGA	PLCKSPWNM	50
Consensus	MVCS.GGM	ALCLIRVEGA	PLCKSPWNM	50
xFrzb	TKMPNHLHHS	TOANAILAIE	QFEGLIQHFC	96
bFrzb	TKMPNHLHHS	TOANAILAIE	QFEGLIQHFC	100
hFRZB	TKMPNHLHHS	TOANAILAIE	QFEGLIQHFC	100
Consensus	TKMPNHLHHS	TOANAILAIE	QFEGLIQHFC	100
xFrzb	QHEPIKPCKS	VCERARQGE	PILIKYRHSW	146
bFrzb	QHEPIKPCKS	VCERARQGE	PILIKYRHSW	150
hFRZB	QHEPIKPCKS	VCERARQGE	PILIKYRHSW	150
Consensus	QHEPIKPCKS	VCERARQGE	PILIKYRHSW	150
xFrzb	AEIVTVEQGT	DSMDFPMD	NGNCG3STAG	196
bFrzb	EAIVTAD-G-	---ADFRMDS	ERCKCKPRA	195
hFRZB	EAIVTAD-G-	---ADFRMDS	ERCKCKPRA	195
Consensus	EAIVTAD-G-	---ADFRMDS	ERCKCKPRA	200
xFrzb	NYVIRAKVKE	VEVKEILKS	SLVNIHREDIV	246
bFrzb	NYVIRAKVKE	VEVKEILKS	SLVNIHREDIV	245
hFRZB	NYVIRAKVKE	VEVKEILKS	SLVNIHREDIV	245
Consensus	NYVIRAKVKE	VEVKEILKS	SLVNIHREDIV	250
xFrzb	PCIVANEVYI	IMGYEDERS	RULLVEGSLA	296
bFrzb	PCIVANEVYI	IMGYEDERS	RULLVEGSLA	295
hFRZB	PCIVANEVYI	IMGYEDERS	RULLVEGSLA	295
Consensus	PCIVANEVYI	IMGYEDERS	RULLVEGSLA	300
xFrzb	-----PRK	SKDPVAPIPN	KNSNSROARN	319
bFrzb	LGLNTSDSSH	SDSTQSQKPG	RNSNSROARN	325
hFRZB	LGLSKSDSSN	SKSTQSQKSG	RNSNPROARN	325
Consensus	LGL..SDSS.	SDSTQSQKPG	RNSNSROARN	330

FIG. 4

*FIG. 5*

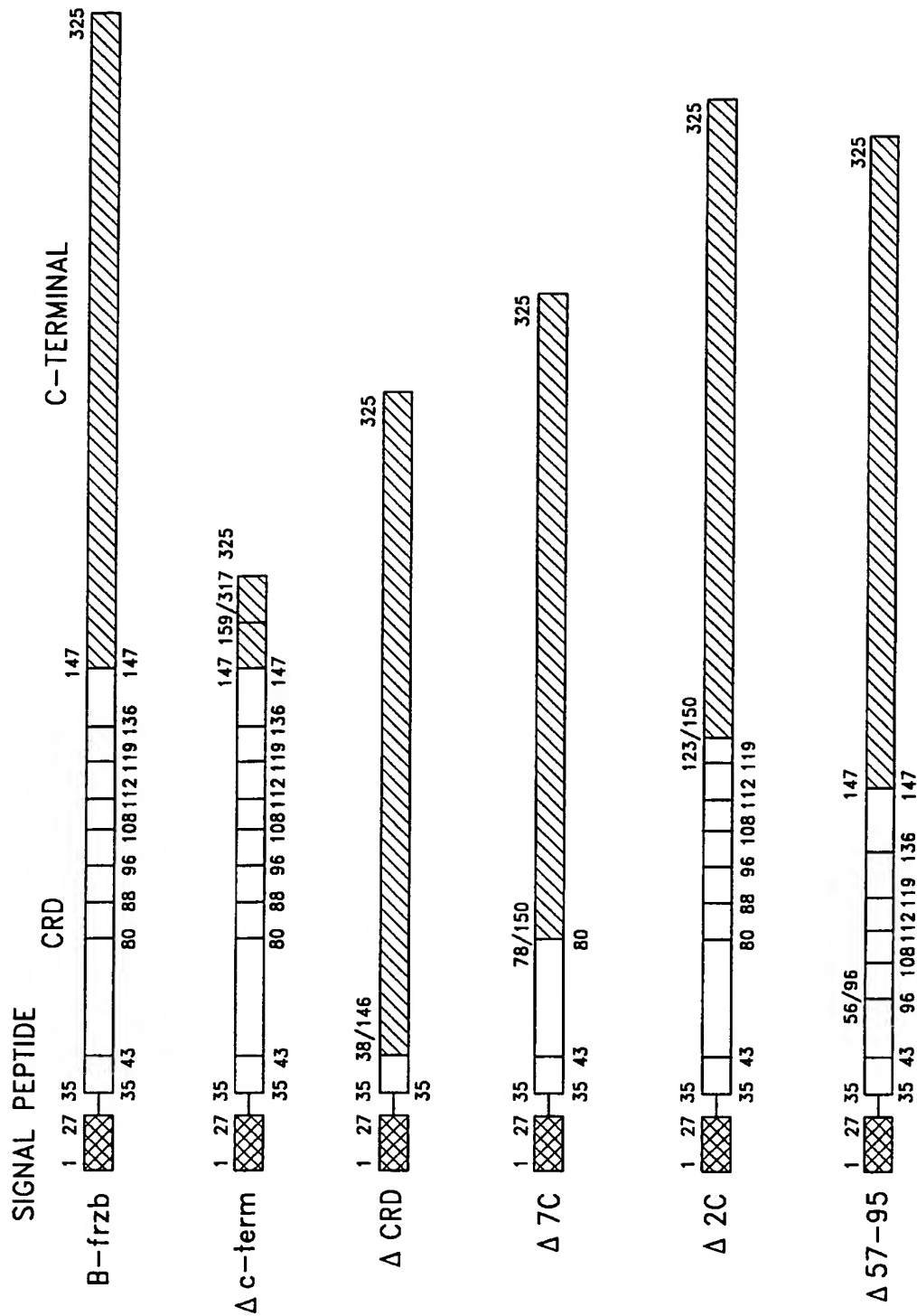
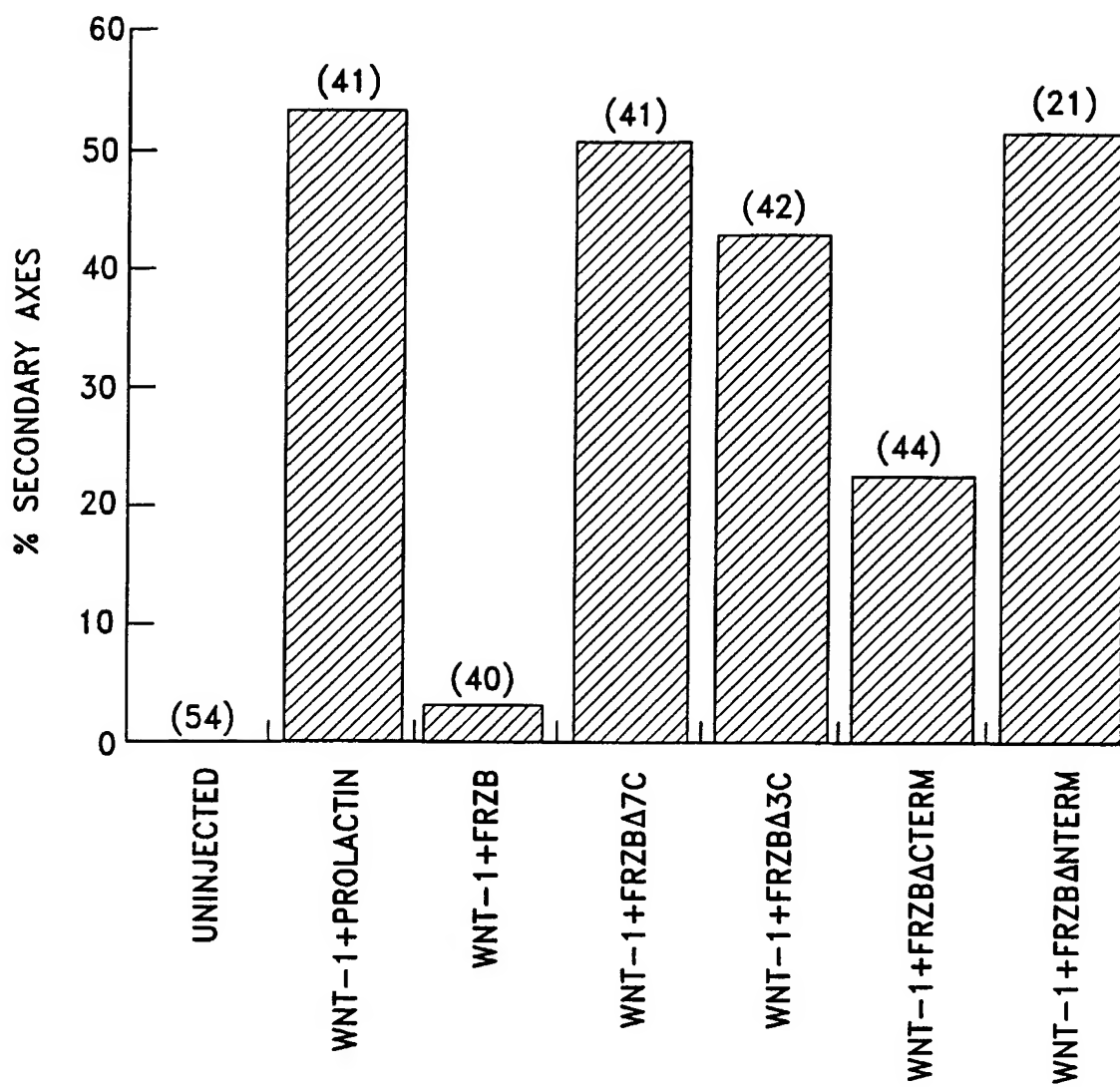


FIG.6

*FIG. 7*

INTERNATIONAL SEARCH REPORT

International / cation No
PCT/US 97/18362

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/71 C07K14/51 C07K16/28 A61K38/18
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 14335 A (US HEALTH ; LUYTEN FRANK P (US); MOOS MALCOLM JR (US); CHANG STEVEN) 17 May 1996 cited in the application see the whole document ---	
A	WANG Y ET AL.: "A large family of putative transmembrane receptors homologous to the product of the Drosophila tissue polarity gene frizzled" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 8, 23 February 1996, pages 4468-4476, XP002056247 cited in the application see the whole document --- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "Z" document member of the same patent family

Date of the actual completion of the international search

20 February 1998

Date of mailing of the international search report

11.03.98

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Authorized officer

Oderwald, H

INTERNATIONAL SEARCH REPORT

International Publication No

PCT/US 97/18362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LUYTEN F P ET AL: "PURIFICATION AND PARTIAL AMINO ACID SEQUENCE OF OSTEOGENIN, A PROTEIN INITIATING BONE DIFFERENTIATION" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 23, 15 August 1989, pages 13377-13380, XP000036793 cited in the application see the whole document ---</p>	
P,X	<p>HOANG B ET AL: "Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis." JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 OCT 18) 271 (42) 26131-7. JOURNAL CODE: HIV. ISSN: 0021-9258., XP002056248 see the whole document ---</p>	1-54
P,X	<p>MAYR T ET AL.: "Human Fritz mRNA, complete cds." EMBL SEQUENCE DATABASE, 3 April 1997, HEIDELBERG, GERMANY, XP002056439 see the whole document ---</p>	2
P,X	<p>MAYR T ET AL.: "Mus musculus Fritz (mfiz) mRNA, complete cds." EMBL SEQUENCE DATABASE, 3 April 1997, HEIDELBERG, GERMANY, XP002056440 see the whole document ---</p>	2
P,X	<p>RATTNER A ET AL.: "A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 94, April 1997, pages 2859-2863, XP002056441 see abstract; figure 1 see page 2859, paragraph 6 - page 2860, paragraph 1 see page 2860, paragraph 5 - paragraph 7 ---</p>	2
E	<p>WO 97 48275 A (UNIV CALIFORNIA) 24 December 1997 see the whole document ---</p>	1-9,11, 13-54
T	<p>WANG S ET AL: "Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8." CELL, (1997 MAR 21) 88 (6) 757-66. JOURNAL CODE: CQ4. ISSN: 0092-8674., XP002056442 see the whole document -----</p>	28-39

INTERNATIONAL SEARCH REPORT

Internatio. application No.
PCT/US 97/18362

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/18362

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 19-39 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/18362

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614335 A	17-05-96	AU 1120295 A	31-05-96
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(54) Title: INJECTABLE FORMULATIONS FOR TREATMENT OF OSTEOPOROTIC BONE (57) Abstract <p>Methods and compositions are provided for the treatment of defects and disease involving osteoporosis, or osteopenic conditions. The methods comprise applying to the site of osteoporotic or osteopenic conditions a composition comprising an active agent, preferably from the TGF-β superfamily of proteins. The advantages of the invention include a reduction or avoidance of the severity and/or incidence of bone fractures. Also, the methods of the present invention are advantageous in that administration is local, rather than systemic.</p>		

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INJECTABLE FORMULATIONS FOR TREATMENT OF OSTEOPOROTIC BONE

FIELD OF THE INVENTION

5 The present invention relates to the field of tissue repair, specifically, to the treatment of osteoporotic bone and/or prevention of osteoporosis. Osteoporotic or osteopenic bone is often characterized by suboptimal bone density. The osteoporotic condition may be related to diet, trauma or stress, or to degenerative or congenital disease. Thus, the present invention may be useful in the treatment and/or the
10 prevention of osteoporosis, or the treatment of osteoporotic or osteopenic bone.

BACKGROUND OF THE INVENTION

Background of the incidence and etiology of need: Idiopathic osteoporosis is a disease of unknown etiology characterized by progressive loss of bone mass and increased fragility. It is a medical problem because it is associated with a marked
15 increase in susceptibility to fractures. It is a public health problem for several reasons. First, it is among the most prevalent of all musculoskeletal disorders. Fifty-six percent of women over 45 years of age are afflicted. Praemer *et al.*, Musculoskeletal Conditions in the United States, American Academy of Orthopaedic Surgeons, Park Ridge, IL (1992). Second, its incidence increases with age. Because the percentage
20 of elderly in the population is increasing, osteoporosis will become more common with time. Third, osteoporosis presently has no known cure, and is difficult to treat locally. Fourth, it is a major economic burden to individuals and to society. Fifth, and most significantly, osteoporosis is associated with a substantial morbidity and mortality. The most serious fracture resulting from osteoporosis is that the of the
25 proximal femur in the region of the hip joint. With an annual incidence of 250,000, hip fractures are currently the most common fracture in the elderly. Praemer *et al.*, *Ibid.* Estimates based on census projections indicate that this figure will increase to approximately 340,000 hip fractures per year by the year 2000. One out of every six caucasian women will have a hip fracture during her lifetime (Cummings *et al.*, Arch
30 Intern Med 149:2455-2458 (1989), and for those who attain the age of 90, this figure becomes one in three.

5 Of the patients who are independent and living at home at the time of hip fracture, approximately 20 percent remain in a long term care institution for at least one year following the fracture. During the first year following injury, the mortality rate is approximately 15% higher than for age and gender matched controls. Praemer *et al.*, *Ibid.* The financial toll of these fractures is also high. In 1988, hip fractures
10 cost the United States 8.7 billion dollars. Praemer *et al.*, *Ibid.* The increased incidence of proximal femur fracture observed in elderly patients is mainly related to two factors: (1) a decreased bone density of their proximal femora; (2) an increased propensity to fall. There is an inverse relationship between the age-related bone loss in the proximal femur and the risk of hip fracture. Each decrease of one standard
15 deviation (SD) in femoral neck bone density increases the age-adjusted risk of hip fracture 2.6 times (95% CI 1.9 - 3.6); and (3) women with bone density in the lowest quartile have an 8.5-fold greater risk of hip fracture than those in the highest quartile. Cummings *et al.*, *The Lancet* 341:72-75 (1993). This relation between hip bone mass and hip fracture risk allows the screening and identification of patients at risk for
20 fracture. Patients who are two standard deviations below peak hip bone mass have passed beneath the "fracture threshold."

Bioactive agents that reduce the incidence of hip fractures or augment the healing of such fractures when they occur would substantially improve the health of the elderly. The availability of an injectable agent would permit its use in fracture
25 prevention, such as hip fracture, without the costs and morbidity of surgical intervention, such as for femoral neck fractures. Such an agent would have application in fracture treatment, without increasing the extent or complexity of the surgical management. The inventors have provided a novel method using bioactive agents to decrease the occurrence and/or severity of fractures to osteoporotic bone,
30 such as an osteoporotic hip.

While several therapies for osteoporosis have been approved, there is none that addresses the localized treatment of proximal femur osteoporosis. Current therapies for osteoporosis are systemic. These include fluoride, bisphosphonates, calcitonin, estrogens and progestins, testosterone, vitamin D metabolites, and/or calcium. In the
35 United States, only estrogens and alendronate, a bisphosphonate, are indicated for the

5 prevention of hip fractures in postmenopausal osteoporotic women. Each of these
agents requires continuous administration over a time period of years. Estrogen has
undesirable side effects (WHO Study Group on Assessment of Fracture Risk and its
Application to Screening for Postmenopausal Osteoporosis. WHO Technical Report
Series 843, Assessment of fracture risk and its application to screening for
10 postmenopausal osteoporosis. Geneva, World Health Organization (1992)),
compliance is poor, and it is not indicated in males. Alendronate requires carefully
separated administration of the drug and sources of calcium and is not recommended
in patients with reduced renal function. A recent study found a reduction in hip
fractures in elderly women with involutional osteoporosis after eighteen months of
15 vitamin D and calcium (Chapuy *et al.* New Engl J. Med. 327:1637-1642 (1992)), but
this treatment has not been conformed by independent sources, and lacks United
States regulatory approval. While several treatments have effectively arrested or
decreased bone loss, only the use of fluoride has been associated with rapid increases
in bone density. However, fluoride treatment increased bone density less in the
20 femoral region as compared to the spine and was associated with poor bone quality.
Hedlund *et al.*, J. Bone Min. Res. 4:223-225 (1989). Fluoride treatment again has not
received regulatory approval. Thus, no safe, effective, generally applicable local
treatment is available which protects against the occurrence of hip fractures in
osteoporosis by providing a reliable and durable increase in hip bone mass.
25 Accordingly, despite substantial endeavors in this field, there remains a need for an
effective method of repair and/or treatment of osteoporotic and osteopenic bone, and
for treatment and/or prevention of osteoporosis.

SUMMARY OF THE INVENTION

30 Accordingly, the present invention provides methods and compositions for
increasing bone mass and quality, and for minimizing or reducing the incidence or
severity of osteoporosis-related fractures. Accordingly, the present invention provides
methods and compositions useful for decreasing the incidence of fractures of
osteoporotic or osteopenic bone. In particular, the present invention comprises
35 methods of treating patients with osteoporosis, or with other evidence of osteoporosis

5 or osteopenic condition. Preferred embodiments where the present invention may prove particularly useful include treatment of metaphyseal bone, including proximal femur (hip), proximal humerus (upper arm), distal radius (wrist), and vertebral bodies (spine), particularly the vertebral body.

10 The method comprises administering to a site of osteopenic or osteoporotic bone, or a site of low bone mass or density, an effective amount of a composition comprising at least one active agent which is capable of inducing growth of bone or increasing the formation of bone tissue or reducing bone loss at the site. Bone mass is commonly designated "bone mineral content" or BMC and is measured in grams. Bone density is commonly designated "bone mineral density" or BMD and is
15 expressed as grams per unit area or grams per unit volume. In a preferred embodiment, the mode of administration is by intraosseous injection. In preferred embodiments, the active agent is one or more proteins selected from the group of proteins known as the Transforming Growth Factors-Beta (TGF- β) superfamily of proteins, preferably selected from the Bone Morphogenetic Proteins (BMPs), the
20 Growth and Differentiation Factors (GDFs), as well as other proteins, as described more fully herein. The methods and compositions of the present invention are advantageous in that they provide a localized treatment for osteoporosis or osteopenic bone, rather than systemic treatment. The present invention is further advantageous in that it utilizes as active agents osteogenic proteins, which may be produced via
25 recombinant DNA technology, and therefore are of potentially unlimited supply. The methods and compositions of the present invention are further advantageous in that regeneration of the bone tissue increases the bone mass/density, increase the bone strength, and thereby reduce the severity of osteoporosis or incidence of osteoporotic lesions, ultimately lessening the incidence of bone fractures.

30 In a preferred embodiment of the present invention, the active agents are administered locally through injection using a suitable buffer and/or carrier. One suitable buffer comprises glycine, sucrose, and glutamic acid hydrochloride, at a pH of less than 6.0. In a preferred embodiment of the invention, this formulation comprises about 2.5% glycine (g/100 ml (w/v)), about 0.5% sucrose (w/v), about 5
35 mM glutamic acid hydrochloride (about 0.1% w/v), and about 0.01% (w/v)

5 polysorbate 80, at a pH of about 4.5. This buffer is referred to later in this application as "MFR 842." Further buffers suitable for use in the present invention are described in United State Patent 5,385,887, the disclosure of which is hereby incorporated by reference. Suitable carriers include collagen gels, hyaluronate, alginates and hyaluronic acids, injectable calcium phosphates, polyols, demineralized bone matrix
10 and combinations of the above. Other carriers which may be useful for the present invention include blood as well as clotting proteins, such as fibrin or thrombin, and oils.

In other preferred embodiments, the active agent further comprises, in addition to one or more proteins selected from the TGF- β superfamily of proteins, one or more
15 auxiliary proteins, such as *Hedgehog*, *Noggin*, *Chordin*, *Frazzled*, *Cerberus* and *Follistatin*, soluble BMP receptors, or other protein or agent, as described further herein.

In addition to healing of osteoporotic bone, compositions of the present invention may be useful for injectable formulations of BMPs for uses such as injection
20 into joints for treatment and repair of osseous defects, cartilage defects, inhibition of cartilage degradation and to promote cartilage repair. The formulations may also be injected into tendons, ligaments and/or their attachment sites to bone. Injectable formulations of BMPs may also find application to other bone sites such as bone cysts, implants into bones, closed fractures and distraction osteogenesis.

25

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, methods and compositions are provided for treatment of patients who exhibit signs of osteoporosis, or osteopenic conditions, including osteoporotic bone lesions. The identification of such patients may be
30 accomplished by procedures which are well known in the art. These procedures include measurement of bone mass/density using dual-energy X-ray absorptiometry (DEXA), Kilgus et al., J. Bone & Joint Surgery, 75-B:279-287 (1992); Markel et al., Acta Orthop Scand, 61:487-498 (1990); and quantitative computed tomography (QCT), Laval-Jeantet et al., J Comput Assist Tomogr, 17:915-921 (1993); Markel,
35 Calcif Tissue Int, 49:427-432 (1991); single-photon absorptiometry, Markel et al.

5 Calcif Tissue Int, 48:392-399 (1991); ultrasound transmission velocity (UTV); Heaney et al., JAMA, 261:2986-2990 (1989); Langton et al., Clin Phys Physiol Meas, 11:243-249 (1990); and radiographic assessment, Gluer et al., J Bone & Mineral Res, 9:671-677 (1994). Other methods of identification of patients at risk of bone fracture include
10 assessment of age-related factors, such as cognisance, as well as prior occurrence of osteoporosis-related fractures. Porter et al., BMJ, 301: 638-641 (1990); Hui et al., J Clin Invest, 81:1804-1809 (1988). The above publications are hereby incorporated by reference herein.

 The methods comprise applying to the osteoporotic or osteopenic site an amount of a composition comprising one or more purified osteogenic proteins which
15 is effective to induce the formation and/or maintenance of bone.

ACTIVE AGENT

 The active agent is preferably selected from the family of proteins known as the transforming growth factors-beta (TGF- β) superfamily of proteins, which includes
20 the activins, inhibins and bone morphogenetic proteins (BMPs). Most preferably, the active agent includes at least one protein selected from the subclass of proteins known generally as BMPs, which have been disclosed to have osteogenic activity, and other growth and differentiation type activities. These BMPs include BMP proteins BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 and BMP-7, disclosed for instance in United States
25 Patents 5,108,922; 5,013,649; 5,116,738; 5,106,748; 5,187,076; and 5,141,905; BMP-8, disclosed in PCT publication WO91/18098; and BMP-9, disclosed in PCT publication WO93/00432, BMP-10, disclosed in PCT application WO94/26893; BMP-11, disclosed in PCT application WO94/26892, or BMP-12 or BMP-13, disclosed in PCT application WO 95/16035; BMP-15, BMP-15, disclosed in United
30 States Patent 5,635,372; or BMP-16, disclosed in co-pending patent application, serial no. 08/715,202, filed on September 18, 1996. Other TGF- β proteins which may be useful as the active agent in the present invention include Vgr-2, Jones et al., Mol. Endocrinol., 6:1961-1968 (1992), and any of the growth and differentiation factors [GDFs], including those described in PCT applications WO94/15965; WO94/15949;
35 WO95/01801; WO95/01802; WO94/21681; WO94/15966; WO95/10539;

5 WO96/01845; WO96/02559 and others. Also useful in the present invention may be BIP, disclosed in WO94/01557; HP00269, disclosed in JP Publication number: 7-250688; and MP52, disclosed in PCT application WO93/16099. The disclosures of all of the above applications are hereby incorporated by reference. A subset of BMPs which are presently preferred for use in the present invention include BMP-2,
10 BMP-4, BMP-5, BMP-6, BMP-7, BMP-10, BMP-12 and BMP-13. The active agent is most preferably BMP-2, the sequence of which is disclosed in United States Patent 5,013,649, the disclosure of which is hereby incorporated by reference. Other BMPs and TGF- β proteins known in the art can also be used.

The active agent may be recombinantly produced, or purified from a protein
15 composition. The active agent, if a TGF- β such as a BMP, or other dimeric protein, may be homodimeric, or may be heterodimeric with other BMPs (e.g., a heterodimer composed of one monomer each of BMP-2 and BMP-6) or with other members of the TGF- β superfamily, such as activins, inhibins and TGF- β 1 (e.g., a heterodimer composed of one monomer each of a BMP and a related member of the TGF- β
20 superfamily). Examples of such heterodimeric proteins are described for example in Published PCT Patent Application WO 93/09229, the specification of which is hereby incorporated herein by reference.

The active agent may further comprise additional agents such as the *Hedgehog*, *Frazzled*, *Chordin*, *Noggin*, *Cerberus* and *Follistatin* proteins. These families of
25 proteins are generally described in Sasai et al., Cell, 79:779-790 (1994) (*Chordin*); PCT Patent Publication WO94/05800 (*Noggin*); and Fukui et al., Developmental Biology, 159:131-139 (1993) (*Follistatin*). *Hedgehog* proteins are described in WO96/16668; WO96/17924; and WO95/18856. The *Frazzled* family of proteins is a recently discovered family of proteins with high homology to the extracellular
30 binding domain of the receptor protein family known as *Frizzled*. The *Frizzled* family of genes and proteins is described in Wang et al., J. Biol. Chem., 271:4468-4476 (1996). The active agent may also include other soluble receptors, such as the truncated soluble receptors disclosed in PCT patent publication WO95/07982. From the teaching of WO95/07982, one skilled in the art will recognize that truncated
35 soluble receptors can be prepared for numerous other receptor proteins. Such would

5 also be encompassed within the present invention. The above publications are hereby incorporated by reference herein.

The amount of active agent useful herein is that amount effective to stimulate increased osteogenic activity of present or infiltrating progenitor or other cells, and will depend upon the size and nature of the defect being treated, as well as the carrier
10 being employed. Generally, the amount of protein to be delivered is in a range of from about 0.1 to about 100 mg; preferably about 1 to about 100 mg; most preferably about 10 to about 80 mg.

CARRIER

Materials which may be useful as the carrier in practicing the present invention
15 include pharmaceutically acceptable materials having viscosity and polarity such that, when added to the bone morphogenetic protein, form a composition that possesses appropriate handling characteristics for injectable application to the site of osteoporotic or osteopenic bone. Adding the carrier to the bone morphogenetic protein allows the protein to remain in the diseased or lesioned site for a time
20 sufficient to allow the protein to increase the otherwise natural rate of regenerative osteogenic activity of the infiltrating mammalian progenitor or other cells, and to form a space in which new tissue can grow and allow for ingrowth of cells. The carrier may also allow the bone morphogenetic protein to be released from the disease or lesion site over a time interval appropriate for optimally increasing the rate of regenerative
25 osteogenic activity of the progenitor cells. The carrier may also supply a framework on which to induce new formation in severely osteoporotic bone.

The most preferred family of carriers comprises collagenous materials. These are preferably in a form suitable for injection, such as a gel. Such gels may be cross-linked or non-cross-linked. Other forms of collagen, such as dispersions or fibrillar
30 collagen, may also be useful in the methods of the present invention. Another preferred family of carriers is cellulosic materials such as alkylcellulose, including hydroxyalkylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, and carboxymethylcellulose, the most preferred being the cationic salts of carboxymethylcellulose (CMC).

5 In the case of cellulosic carriers and collagen gels, it is preferred that the carrier be in the form of a hydrated cellulosic viscous gel. Viscosity may be increased through mechanical means, such as high agitation for a suitable period of time, followed by autoclaving, or chemically. The active agent and cellulosic carrier is preferably in a solution of suitable buffer. One preferred buffer solution is a
10 composition comprising, in addition to the active agent, about 1.0 to about 10.0% (w/v) glycine, about 0.1 to about 5.0% (w/v) of a sugar, preferably sucrose, about 1 to about 20 mM glutamic acid hydrochloride, and optionally about 0.01 to about 0.1% of a non-ionic surfactant, such as polysorbate 80. Preferred solutions are from about 1% to about 20% w/v cellulosic carrier/buffer. If desired, a salt may be added. A
15 preferred viscous gel carrier is described in Example 2 below. The amount of osteogenic protein useful with viscous gel carrier is generally in a range of from about 0.1 to about 100 mg, preferably about 1 to about 100 mg; most preferably about 10 to about 80 mg per cubic centimeter of implant material required.

 Another class of materials of particular interest for injectable carriers are
20 resorbable hydroxyapatites as well as minerals, ceramics and phosphates. Resorbable hydroxyapatites, for example, can be formulated at various porosities with varying resorption rates; their handling characteristics vary from hard implantable types, to gel-like consistency, to those that are injectable but harden at body temperature. Suitable hydroxyapatite and ceramic carriers are described, for example in
25 WO96/36562; and United States Patents 5,543,019; 5,306,305; 5,258,044; 5,496,399; 5,455,231; 5,336,264; 5,178,845; 5,053,212; 5,047,031; 5,129,905; 5,034,059; 4,880,610; 5,290,763; and 5,563,124; the disclosures of which are incorporated herein by reference.

 Another preferred family of carriers for administration of the active agent of
30 the present invention are injectable polymers, which may be viscous and which may optionally include a sequestering agent as well. Suitable polymers and sequestering agents include those described in United States Patent 5,171,579, the entire disclosure of which is incorporated herein by reference. Other polymers include the pluronics, such as Poloxamer 407 gel. Pluronics are a class of water soluble ABA type block
35 surfactant copolymers which exhibit the unique property of reverse thermal gelation.

5 They are liquid (and hence syringeable) at 4°C and gel at body temperature. Poloxamer 407, MW 12,500, is excreted unchanged in the urine after systemic absorption and has supposedly been shown to be non-toxic in animals. Polylactides and/or polyethylene glycols, including poly(lactide)/poly(ethylene glycol) gels. Polylactides may be dissolved in polyethylene glycols, such as low molecular weight
10 (2000) PLA dissolved in PEG to produce a syringeable solution that precipitates PLA upon injection into an aqueous environment, resulting in a relatively firm gel. In addition, the literature cites conjugates, such as Poly(lactic acid)-poly(ethylene glycol) conjugates, as appropriate carriers for BMPs (Miyamoto *et al.*, Clin. Orthop. Rel. Res. 294:333 (1993)). Also useful as the carriers are fibrin-based polymers, in liquid or gel
15 form. Among the materials useful as sequestering agents are hyaluronic acid, sodium alginate, poly(ethylene glycol), polyoxyethylene oxide, carboxyvinyl polymer and poly(vinyl alcohol), and cellulosic materials, such as hydroxycelluloses. One such preferred agent is carboxymethylcellulose.

The above materials disclosed to be useful as sequestering agents may
20 themselves be useful as carriers for injection. In addition, combinations of the above described materials may be used.

It is also possible to use as injectable carriers solid materials which are narrow enough to be administered through injection. In such cases, the solid material may be combined with a viscous liquid carrier for ease and uniformity of administration.

25 In cases where the carrier may be of higher viscosity than optimal, the carrier may optionally be combined with a diluent, such as aqueous glycerol, preferably the carrier diluent would be present in concentrations of about 10 to about 80% (v/v). Also, the above materials may be combined in particular embodiments of the present invention. For example, polymers, such as porous particulate polymers, may be
30 dissolved or suspended in cellulosic or gel carriers to increase viscosity.

In a preferred embodiment of the present invention, the active agents are administered locally through injection using only a suitable buffer as carrier. One suitable buffer comprises glycine, sucrose, and glutamic acid hydrochloride, at a pH of less than 6.0. Preferred compositions of buffer solutions comprise about 1.0 to
35 about 10.0% (w/v) glycine, about 0.1 to about 5.0% (w/v) of a sugar, preferably

5 sucrose, about 1 to about 20 mM glutamine, glutamic acid, or glutamic acid hydrochloride, and optionally about 0.01 to about 0.1% of a non-ionic surfactant, such as polysorbate 80. In a preferred embodiment of the invention, this formulation comprises about 2.5% glycine (g/100 ml (w/v)), about 0.5% sucrose (w/v), about 5 mM glutamic acid hydrochloride (about 0.1% w/v), and about 0.01% (w/v)
10 polysorbate 80, at a pH of about 4.5. This buffer has been described as MFR 842. Further buffers suitable for use in the present invention are described in United State Patent 5,385,887, the disclosure of which is hereby incorporated by reference. Preferred solutions may also include combinations of buffer and other carrier, such as a combination of buffer and cellulosic carrier. Preferred ranges for this combination
15 are from about 1% to about 20% w/v cellulosic carrier/buffer. If desired, a salt may be added.

MODE OF ADMINISTRATION

The injectable compositions of the present invention may be administered in any clinically acceptable manner of injection. A number of commercially available
20 syringes may be suitable for use in the present invention, and for administration of the compositions of the present invention. For example, suitable syringes are available the Calasept^R syringe [JS Dental Manufacturing, Ridgefield CT] comprises sterile calcium hydroxide paste in isotonic saline solution, in a non-aspirating or modified aspirating cartridge syringe; Henke-Ject Aspirating Syringe and Hypo Brand Dental
25 Syringes/Needles [Smith & Nephew MPL, Franklin Park, IL]; intraosseous needles from MPL, Inc., Chicago IL; and Luer-Lok^R Syringes [Becton Dickinson, Franklin Lakes, NJ], may all be appropriate syringes for use in the present invention.

In another embodiment of the present invention, bone morphogenetic proteins are used as an osteoinductive agent in the process known as distraction osteogenesis.
30 This process is an alternative to segmental bone regeneration in response to implanted osteoinductive agents. In traditional segmental bone repair, the osteoinductive agent and carrier are placed in the defect created between the parent bone ends. For bone formation to occur, the osteoinductive agent has to have sufficient residence time in the defect to stimulate differentiation of sufficient numbers of bone forming cells to
35 support new bone formation. The carrier also has to have sufficient residence time to

5 serve as a scaffold for bone forming cells to attach (osteoconduction). In addition, the carrier also has to have sufficient porosity to allow cells and blood vessels to penetrate into the defect for bone formation to occur. In contrast, the process of distraction osteogenesis creates a regenerate construct between the distracted parent bone ends that is highly vascular and contains a large population of mesenchymal stem cells
10 destined to become bone forming cells. As a result, the regenerate construct represents a much more ideal environment for cell differentiation growth factors such as rhBMP-2 to stimulate rapid bone induction relative to induction of bone within a segmental defect.

 The process of distraction osteogenesis begins with an initial latency period
15 allowing a fibrous connection to form between the bone ends to be distracted. Following this latency period, the bone ends are slowly distracted at a controlled rate of up to 1 mm per day in human clinical cases. Once the regenerate forms and the bone ends are distracted to the appropriate length, a prolonged consolidation period is required to allow the regenerate to form bone. This prolonged consolidation period
20 which can be on the order 4 to 6 months is associated with considerable morbidity. A frequent complication is the occurrence of pin track infections resulting from the extended length of time the external fixator used to generate the distraction must remain in place. In addition, there are considerable psychological effects and life style alterations associated with wearing the external fixator for prolonged periods of time.
25 In addition to complications associated with the external fixator, there are a number of patients where the regenerate does not form properly and a delayed union or non-union occurs. Since the regenerate contains a responsive cell population and is already highly vascularized following the initial distraction phase, the use of bone morphogenetic proteins may rapidly accelerate the rate of bone formation during the
30 normally prolonged consolidation phase of distraction osteogenesis. Acceleration of the distraction phase is limited by stretching of the soft tissues associated with bone. The cells created using distraction osteogenesis may also be harvested in order to provide a source of cells which are primed for osteogenesis. These cells can be cultured to prepare immortalized cell lines. If desired, these cells can also be
35 immunotolerized using agents such as CTLA4 receptors [U.S. Patent 5,434,131] or

5 CTLA4 ligands or B7 monoclonal antibodies [WO 96/40915]. Methods and materials for such immunotolerization are disclosed in the above references, and include co-transfection or treatment with these factors. The disclosure of these references is hereby incorporated herein by reference.

10 The following examples further describe the practice of embodiments of the invention with rhBMP-2 in a buffer carrier. The examples are not limiting, and as will be appreciated by those skilled in the art, can be varied in accordance with the above specification.

EXAMPLES

Example 1: Oophorectomized Rat Model:

15 The oophorectomized rat is a well-established model of osteoporosis and its use is recommended by the FDA as one of the animal models to be employed in demonstrating efficacy of potential therapeutic agents for osteoporosis. Preclinical studies testing the efficacy of rhBMP-2 in the oophorectomized rat model of
20 osteoporosis demonstrated that 100 μ g rhBMP-2 in MFR 842 buffer, injected into the intramedullary space of the tibial shaft, was associated with extensive bone formation. Further evaluation with this model is conducted at lower doses.

25 In a similar study, five ovariectomized rats were injected with 40 μ g of rhBMP-2 in MFR 842 buffer solution into one side of the femoral neck, and MFR 842 buffer alone on the other side. After six weeks, the femora are harvested, analyzed by DEXA for bone density and by histology.

30 In a larger study, six weeks after ovariectomy, rat tibiae are injected with 0, 10, or 50 μ g of rhBMP-2 in 20 μ L MFR 842 buffer. Controls include sham ovariectomized animals [surgery performed, but ovaries are not removed], buffer injections and sham surgeries. After six weeks, the femora are harvested and groups analyzed by DEXA, biomechanics, and histomorphometry.

Example 2. Sheep Core Decompression Model

35 Preclinical studies in support of the application of rhBMP-2 to the treatment of osteonecrosis of the hip at the time of core decompression demonstrated that

5 rhBMP-2 stimulates bone formation in the core decompression track within the femoral neck of normal (non-osteoporotic) sheep. In this study, defects in both the femoral head/neck and distal femur were created, the latter to facilitate DEXA imaging. rhBMP-2 in various formulations, including buffer, blood, blood clot and collagen dispersions was placed at the site of defect.

10 **Example 3. Human Core Decompression Study.**

Patients with osteonecrosis of the femoral head were treated by core decompression with or without implantation of blood clot containing rhBMP-2 into the core track. Patients who received rhBMP-2 experienced a reduction in the volume of necrotic bone compared to patients treated by core decompression alone. The treatment was well tolerated with complications limited to some bone formation adjacent to the cortical entry site.

15 **Example 4. Biodistribution of rhBMP-2.**

Four aged ewes underwent surgical placement of a cannulated needle into the femoral neck using uniplanar fluoroscopic guidance. A bolus injection of radiolabelled rhBMP-2 in buffer solution was delivered through the needle into the proximal femur. Local (bone) distribution of rhBMP-2 was monitored by gamma camera. Systemic distribution was assessed by periodic serum sampling. *In vivo* gamma camera images of the femur indicate that the injected rhBMP-2 enters the femoral head and neck and that part of the injected material is retained at these sites for at least 72 hours. Serum analysis of radiolabelled rhBMP-2 indicates that the majority (~85%) of the rhBMP-2 enters the systemic circulation within five minutes of administration.

25 **Example 5. Intra-Femoral Injections.**

Patients with osteoporosis who have sustained a fracture of the proximal femur are identified. Intraosseous administration of injectable rhBMP-2 is accomplished in the non-fractured (contralateral) femur using an operating room, biplanar fluoroscopy, and patient positioning on a fracture table. Each patient undergoes the necessary surgical treatment of the fractured femur. At the completion of surgical procedure, but before termination of the anesthesia, the lower extremities are repositioned on the fracture table to gain access to the uninjured contralateral hip. Under sterile

5 conditions and biplanar image intensifier visualization, the designated dose of rhBMP-2 is delivered by percutaneous, intraosseous route into the proximal femur. Follow-up evaluations include radiographic assessment, clinical evaluation of infection and fracture, and DEXA evidence of bone formation.

Example 6: Rabbit Ulnar Osteotomy and Fracture Models.

10 In the osteotomy model, the ulna is simply exposed, the periosteum is stripped, and a 2 to 5 mm osteotomy created using an oscillating saw. The incision is then closed, and the appendage bandaged to provide additional stability. Bilateral surgeries may be used in order to compare active agent against untreated or control substances.

The rabbit ulnar fracture model is similar to the rat femoral fracture model.
15 The rabbit ulnar fracture model relies on a weight-driven blunt instrument to create the fracture essentially using incident three-point bending. The fracture is created by fully extending and clamping the forelimb to an aluminum rod containing two vertical bars. In a modification from the rat apparatus, a 23G needle is placed in between the radius and ulna to serve as a guide pin. The blunt guillotine is then positioned, and
20 a 850 gm weight raised to 45 cm and allowed to fall to create the fracture.

Assessment of efficacy in both the rat femoral and rabbit ulnar fracture models can be accomplished primarily using biomechanics. Qualitative measures of fracture repair, such as radiology, can be obtained, but it is difficult to see subtle differences over normal fracture repair, and will not give a convincing demonstration that the
25 injection is translating to accelerated fracture repair.

Example 7: Rat Ectopic Implant Models

In one experiment, several non-collagenous materials were tested in the rat ectopic implant model. The materials included in this study were:

1. MFR 842 buffer;
- 30 2. Poloxamer 407 gel.
3. Poly(lactide)/poly(ethylene glycol) gels. Low molecular weight (2000) PLA dissolved in PEG produces a syringeable solution that precipitates PLA upon injection into an aqueous environment, resulting in a relatively firm gel. Two different percentages were tested.
- 35 4. Poly(lactic acid)-poly(ethylene glycol) conjugates.

5 Each material was implanted with 0, 10, and 80 µg rhBMP-2. Lyophilized rhBMP-2 was mixed with the gels using a mortar and pestle. The buffer, Pluronic gels, and PEG/PLA gels were first injected into blood clots. The PLA-PEG conjugates were implanted directly into the subcutaneous space. Six implants (three animals) were used per group. In addition, the intramuscular injection technique was
10 investigated using the PLA/PEG gel and one Pluronic gel (both at the high dose only).

 The above were scored for bone formation. Bone score is a semi-quantitative measurement of the area of bone present in representative histological sections; the score ranges from 0 for no bone present, to 5 when the entire section consists of bone. Coupled with the wet weights and qualitative histological assessment of the sections,
15 the following conclusions can be made. The Pluronics produced relatively little bone at the lower dose of rhBMP-2, and induced none when placed intramuscularly. Presumably the material does not have a long enough residence time, or does not sequester rhBMP-2 sufficiently. In addition, a significant inflammatory response was noted. The PLA/PEG gels produced reasonable quantities of bone, with some residual
20 matrix observed (presumably the precipitated PLA) along with a foreign body giant cell response. The PLA-PEG conjugates produced well-defined ossicles of bone with bone marrow. Of the gels tested, this material performed best. Based on the samples tested, the intramuscular implantation appeared to be a more rigorous test of materials. In this experiment, rhBMP-2 in buffer simply injected into blood clot performed the
25 best overall; presumably the rhBMP-2 is able to rapidly distribute and bind to the blood clot.

 In another rat ectopic study, several cross-linked and non-cross-linked collagen materials were tested. In addition, porosity was introduced into the PLA/PEG gels by the addition of MFR 842 buffer as an excipient, and a lower percentage of PLA was
30 tested. Some of the collagen materials were tested in an aqueous (MFR 842) or non-aqueous (PLA/PEG gel) environment. The collagen materials tested included:

1. Homogenized Helistat® bovine type I collagen cross-linked hemostatic sponge (currently in use with rhBMP-2 clinically) made into a suspension;
2. Homogenized Helitene™ (fibrillar form). Only tested with PLA/PEG in this
35 experiment;

5 3. Collagen dispersion (Integra LifeSciences, Plainsboro, NJ). The non-cross-linked parent material of Helistat®. At the concentration tested (0.78%, as provided), it is not viscous at all.

 4. Zimmer collagen gel. The collagen component of the Collagraft® kit.

10 All materials were tested with 10 µg rhBMP-2 using the blood clot injection method. The samples were scored for histologic bone formation. The materials which performed best in this study were the Helistat® dispersion and the collagen gel, yielding bone scores of 3 to 4, and no adverse tissue reaction. All PLA/PEG containing materials showed much smaller amounts of bone, and significant inflammatory response (non-specific foreign body type). Addition of PLA/PEG to the
15 collagen material decreased the bone formation observed. In this experiment, the rhBMP-2 in buffer placed in blood clot perform poorly; this may be because a larger gauge needle was used to place the material, and it flowed back along the needle track before it could distribute in the blood clot. Likewise, the ILS dispersion performed poorly, perhaps due to low viscosity. These results indicate relatively little promise
20 for the PLA/PEG gels, unless use of very low molecular weight PLA accelerates its resorption time. The collagen materials stand out above the other materials tested to date using this rat ectopic assay system.

 Various collagen materials tested include 2% ILS dispersion, 2% Helitene™ dispersion, 2% Helistat® dispersion, the Zimmer collagen gel, and another collagen
25 gel (6.5%) from Matrix Pharmaceuticals. All have been placed at 0, 20, and 80 µg rhBMP-2 and analyzed histologically.

Example 8: Distraction Osteogenesis

 BMP-2 may accelerate the consolidation (bone formation or mineralization)
30 phase in a rabbit model of limb lengthening. Osteotomies are created in rabbit tibia. The tibia are then be distracted 2 cm over a period of approximately ten days. Following the distraction phase, rhBMP-2 is administered. Two modes of application are tested. One set of animals has rhBMP-2/ACS (1.5 mg/mL; 0.075 mg/tibia) surgically placed within the regenerate. The second group of animals has
35 rhBMP-2/MFR842 buffer (0.75 mg/mL; 0.075 mg/tibia) percutaneously injected into

5 three sites within the regenerate. A third group of control animals has surgical
intervention similar to the rhBMP-2/ACS-treated animals, with placement of
ACS/buffer within the regenerate. Additional sets of animals have distraction
performed, with no surgical intervention. Groups of animals are sacrificed at 5, 14
and 24 days. Radiographs are taken following the distraction phase and at sacrifice
10 and are used to evaluate new bone formation in response to treatment and control.
Bone density within the defect is determined with Dual Energy X-Ray Absorptiometry
and Peripheral Quantitative Computed Tomography (pQCT). The three dimensional
distribution of bone within the defect is evaluated with pQCT. Histological evaluation
of non-decalcified specimens is used to quantify histomorphometric parameters.

15 Results to date indicate a visually detectable acceleration of bone induction in
rhBMP-2/ACS treated animals compared to untreated surgical controls at 5 days and
14 days based on radiographic evaluation. The DXA and pQCT images confirm these
findings. In addition the pQCT images indicate greater three-dimensional distribution
of bone within the regenerate and evidence of early stages of corticalization in the
20 rhBMP-2/ACS treated animals at 14 days. Further results may support the
rhBMP-2/ACS treatment effect of significant acceleration of the consolidation phase
of distraction osteogenesis. Formation of an intact cortex is the primary indicator for
removal of the external fixator and associated return to unprotected full weight
bearing. The rhBMP-2/MFR842 injection buffer treatment is the preferred route of
25 administration having more wide spread application in primary distraction
osteogenesis.

5

CLAIMS

We claim:

1. A method for reducing the severity and/or incidence of fractures comprising administering to a site of osteoporotic or osteopenic bone tissue an injectable composition comprising an effective amount of an active agent in a suitable carrier.
- 10 2. The method of claim 1, wherein the active agent is a member of the TGF- β superfamily of proteins.
3. The method of claim 1, wherein the active agent is a member of the bone morphogenetic protein family of proteins.
4. The method of claim 1, wherein the active agent is selected from the group
15 consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-10, BMP-12 and BMP-13.
5. The method of claim 1, wherein the active agent is BMP-2.
6. The method of claim 1, wherein the carrier is selected from the group consisting of a suitable buffer, blood, collagen gel and injectable calcium phosphates.
- 20 7. A method for healing bone fractures comprising implanting BMP into the region between ends of the bone fracture and allowing such rhBMP-2 to stimulate bone induction.
8. A cell produced by the method of claim 7 and harvested from the region between bone ends.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/01143

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/08 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 5 597 897 A (RON EYAL ET AL) 28 January 1997 * see in particular examples 4,5 and col. 5, l. 58- col.6, l.7 *	1-7
X	& WO 93 00050 A (GENETICS INST.) 7 January 1993 ---	1-7
X	US 5 453 419 A (MURAKAMI KAZUO ET AL) 26 September 1995 *see in particular col. 7, l.63 - col. 8, l. 12, and claims 1-2* ---	1-7
X	US 5 166 058 A (WANG ELIZABETH A ET AL) 24 November 1992 *see in particular col.5 l. 50 - col.7, l.43 * --- -/--	1-7



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

20 May 1998

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/01143

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WANG: "Bone morphogenetic proteins (BMPS): Therapeutic potential in healing bony defects"</p> <p>TRENDS IN BIOTECHNOLOGY, vol. 11, no. 9, 1993, pages 379-383, XP002065499</p> <p>* see in particular p.381, 2nd paragraph -382 *</p>	1-7
X	<p>REDDI ET AL.: "Osteoporosis (chapter 9, p. 281-287)"</p> <p>1996 , ACADEMIC , SAN DIEGO, CALIF. XP002065501</p> <p>*see in particular p. 285 *</p>	1-7
X	<p>TAGAKI ET AL.: "The role of bone marrow in bone morphogenetic protein-induced repair of femoral massive diaphyseal defects"</p> <p>CLINICAL ORTHOPAEDICS AND RELATED RESEARCH, vol. 171, 1982, pages 224-231, XP002065500</p> <p>* see in particular p. 230 (summary) and Figures 1-5 n*</p>	1-3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 01143

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 8
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claim 8 relates to a cell "produced by the method of claim 7". However, claim 7 does not describe such a method but relates to a method of treatment.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/01143

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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